DEVELOPMENTAL AND GONADAL REGULATION OF HYPOTHALAMIC-PITUITARY-ADRENAL AXIS FUNCTION IN ADOLESCENT AND ADULT RATS

Matthew R. Green

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Department of Psychology
BROCK UNIVERSITY
St. Catharines, Ontario

Abstract

The hypothalamic-pituitary-adrenal (HPA) axis regulates the release of stress hormones and its function is dependent on various factors including prior exposure to stressors, circulating gonadal hormones, and developmental status. The overarching goal of this thesis was to uncover the potential mechanisms mediating developmental changes in HPA function and its regulation by gonadal hormones during adolescence and early adulthood. In Chapter 2, I found that pre-pubertal (postnatal day [P]35) and post-pubertal (P45) adolescents responded to an acute stressor with greater release of corticosterone (the main stress hormone in rodents) compared with adults (P75). To determine whether differences in corticosterone release were related to ongoing maturation of HPA feedback, I investigated glucocorticoid receptor (GR) activity and mRNA expression of receptors (*Nr3c1*, *Nr3c2*) and their co-chaperones (*Fkbp5*, *Fkbp4*, *Bag1*) in the hippocampus. I provide novel evidence that P35 males have more, not less, GR translocation from the cytosol to the nucleus in response to stress compared with P75 males. Gene expression remained relatively stable across development, except for *Fkbp4*, which codes for a pro-translocation protein and was up-regulated in P35 males relative to expression in P75 males. Thus, there are developmental shifts in the hormonal response to stress that are likely unrelated to GR activity in the hippocampus. In Chapter 3, I investigated whether differences in HPA function are explained by gonadal status; in adult males, testosterone reduces HPA function. Age-related differences in corticosterone release persisted when orchiectomized (OCX) males at each age were administered testosterone. Moreover, the effect of testosterone changed across the adolescent period; relative to those that got blank implants, testosterone had no effect on post-stress

concentrations of corticosterone at P35, increased concentrations at P45, and tended to reduce concentrations at P75. Testosterone reduced expression of AVP in the PVN at all ages, but did not affect Fos (a marker of neuronal activation) expression. I hypothesized that the age-specific effects of testosterone on corticosterone were related to differential conversion to metabolites (e.g., estradiol), which I tested using androgen receptor (AR) and estrogen receptor (ER) antagonists (flutamide and tamoxifen, respectively) in the presence of testosterone or dihydrotestosterone (DHT). Testosterone produced a similar, albeit non-significant, age-specific pattern of effects on corticosterone as described above, and I found little evidence for effects of receptor antagonists. Androgens reduced post-stress concentrations of progesterone in all age groups, and flutamide prevented the effect. Together, this study provides evidence for developmental shifts in stress responses and their regulation by gonadal hormones. In Chapter 4, I examined the influence of estradiol on HPA function in adult female rats as a first step toward understanding developmental shifts. Ovariectomy (OVX) reduced post-stress concentrations of corticosterone compared with sham OVX and OVX females given estradiol alone or in combination with progesterone. I also found that OVX females had greater cytosolic expression of GR, possibly increasing sensitivity to corticosterone. In a second experiment, I found that progesterone partially mitigated the effect of estradiol on corticosterone release and that gene expression of stress hormone receptors (*Nr3c1*, *Nr3c2*), their co-chaperones (*Fkbp5*, *Fkbp4*, *Bag1*), and a co-activator (*Src-1*) did not change as a function of ovarian hormones. Together, these studies build on previous research investigating developmental and gonadal regulation of HPA activity and provide novel findings regarding potential mechanisms underlying their actions.

Keywords: stress; glucocorticoid receptor; negative feedback; co-chaperones; gonadal hormones

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Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

ACTH (adrenocorticotropic hormone): a hormone that is released from cells in the anterior pituitary in response to CRF and causes the release of corticosteroids from the adrenals.

AR (androgen receptors): the principle receptor for androgens (e.g., testosterone and DHT) that acts primarily as a transcription factor.

AVP (arginine vasopressin): a neuropeptide that is released from cells in the PVN in response to stress and that potentiates the release of ACTH from the anterior pituitary.

BNST (bed nucleus of the stria terminalis): a brain region that relays stress-related information from other limbic regions (e.g., mPFC, ventral subiculum, amygdala) to the PVN and has modulatory influences on its activity.

Bag1 (Bcl-2 associated athanogene 1): the gene that codes for Bag1 protein.

Bag1 (Bcl-2 associated athanogene 1): a co-chaperone protein that negatively regulates GR activity, likely by inhibiting its assembly, translocation, and transcriptional properties.

CBG (corticosteroid binding globulin): a transport protein that binds glucocorticoids for distribution throughout the body, and limits their availability to tissues.

CRF (corticotropin releasing factor): a neuropeptide that is released from cells in the PVN in response to stress and that causes the release of ACTH from the anterior pituitary.

DHT (dihydrotestosterone): a potent androgen that, unlike testosterone, cannot be converted to estradiol via aromatase.

ER (estrogen receptors): the principal receptor target for estrogens, which come in several sub-types (ERα and ERβ) that act primarily as transcription factors.

Fkbp4 (FK506 binding protein 4): the gene that codes for Fkbp52 protein.

Fkbp5 (FK506 binding protein 5): the gene that codes for Fkbp51 protein.

Fkbp52 (FK506 binding protein 51): a co-chaperone protein that binds to GR and reduces its affinity for its corticosteroids and ability to translocate into the nucleus.

Fkbp52 (FK506 binding protein 52): a co-chaperone protein that binds to GR and promotes translocation by recruiting the motor protein dynein.

GDX (gonadectomy): a surgical procedure to remove the gonads (i.e., ovaries or testes), which is main site of sex hormone production (i.e., estrogens and androgens).

GR (glucocorticoid receptor): one of the two main receptors for stress hormones (i.e., corticosteroids) that acts primarily as a transcription factor.

GRE (glucocorticoid response elements): a region on the DNA where GR can bind to alter gene transcription.

HPA (hypothalamic-pituitary-adrenal) axis: the system that controls the release of stress hormones.

HPG (hypothalamic-pituitary-gonadal) axis: the system that controls the release of gonadal hormones.

mPOA (medial preoptic area): a hypothalamic region that is primarily known for its involvement in sexual behaviour, and that also modulates HPA activity.

MR (mineralocorticoid receptor): one of two main receptors for stress hormones (i.e., corticosteroids) that acts primarily as a transcription factor.

mRNA (messenger ribonucleic acid): a product of gene expression (i.e., transcription) that contains the information for making a protein.

Nr3c1 (nuclear receptor subfamily 3 group 1): the gene that codes for GR protein.

Nr3c2 (nuclear receptor subfamily 3 group 1): the gene that codes for MR protein.

OCX (orchiectomy): a surgical procedure to remove the testes (the main source of endogenous androgen production).

OVX (ovariectomy): a surgical procedure to remove the ovaries (the main source of endogenous estrogen production and a source for progesterone).

POMC (pro-opiomelanocortin): a precursor poly-peptide that produces various peptides when cleaved by enzymes, including ACTH.

PVN (paraventricular nucleus): the region in the hypothalamus that regulates HPA axis activity by integrating stress-related input and releasing CRF and AVP.

Src-1 (steroid receptor co-activator 1): the gene that codes for Src-1 protein.

Src-1 (steroid receptor co-activator 1): a protein that interacts with nuclear receptors (e.g., GR) and that regulates transcriptional activity by increasing accessibility to DNA.

Chapter 1: General Introduction

This chapter has been adapted from a published article:

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Introduction

Despite the diverse functions of the hypothalamic–pituitary–adrenal (HPA) axis, it is best known for its role in the stress response. When confronted with stressors, the HPA axis initiates a cascade of signaling that culminates in increased release of glucocorticoids from the adrenal glands, which helps an organism meet the demands of its environment (Sapolsky et al., 2000). Although acute activation of the HPA axis is adaptive, repeated or prolonged activation produces allostatic load and can have deleterious effects on physiology and function (Juster et al., 2010). There is much evidence for differences in HPA function from prenatal life to old age (Lupien et al., 2009), and for sex differences in HPA function (Bangasser & Valentino, 2014; Panagiotakopoulos & Neigh, 2014). Relative to research efforts on the perinatal and adult periods of development, however, far less is known about the HPA axis during adolescence. Even less is known regarding the regulation of the HPA axis by the hypothalamic–pituitary–gonadal (HPG) axis during this time. In this review, we outline what is known from investigations in rats about how gonadal hormones organize and modulate HPA activity at different stages of development, and then focus on adolescence, highlighting the key outstanding questions regarding HPG–HPA interactions during that transitional time of life. Fig. 1-1 contains the key terms and abbreviations used in the review.

HPA signaling

The neuroendocrine stress response is initiated in the medial parvocellular portion of the paraventricular nucleus (PVN) of the hypothalamus. Excitatory input to the PVN is derived predominantly from ascending brainstem pathways that respond to physiological

challenges (e.g., ether inhalation and hypotension) and from limbic forebrain regions such as the amygdala, which activate the PVN through a multi-synaptic pathway during exposure to psychological challenges (e.g., restraint or conditioned fear) (Herman & Cullinan, 1997; Myers et al., 2014). The release of corticotropin releasing factor (CRF) and arginine vasopressin (AVP) into the hypophyseal portal veins to the anterior pituitary triggers the release of adrenocorticotropic hormone (ACTH) into general circulation, which then stimulates the synthesis and release of glucocorticoids (primarily corticosterone in rats) from the adrenal cortex. The release of glucocorticoids is regulated by a negative feedback system that operates at all levels of the axis as well as at upstream brain regions (notably, the hippocampus and medial prefrontal cortex), which dampen further HPA activation and promote a return to basal titers (Herman et al., 2005). In the absence of stressors, basal concentrations of glucocorticoids are lower and release from the adrenal cortex occurs in pulses (~1 per hour). The extent of release is influenced by the circadian rhythm, with the peak and nadir corresponding to the beginning and end of the active phase each day, respectively.

Glucocorticoids help an organism meet the demands of its environment by increasing energy metabolism by enhancing gluconeogenesis and glycolysis (reviewed in Sapolsky et al. 2000). Moreover, glucocorticoids can potentiate glucagon signaling and reduce insulin signaling, leading to greater release of glucose from the liver. During times of stress, greater glucose trafficking to the brain and muscles helps an organism cope with the challenge. Glucocorticoids released in response to threats will also constrain immune function and have anti-inflammatory activity, protecting an organism from overshooting an immune and HPA response (immune challenges activate HPA activity).

One means by which increased glucocorticoid signaling restricts immune function is by dampening lymphocyte production and the synthesis and release of cytokines. Not all actions of glucocorticoids involve stress-related concentrations, however, as resting concentrations have permissive roles on stress reactivity, such as enhancing the effects of catecholamines on increasing cardiovascular tone. When glucocorticoids are elevated (e.g., after stress), non-essential behaviours (e.g., reproduction and feeding) are inhibited, and the consolidation of emotionally-salient information is enhanced, which allows the organism to encode information necessary for survival and to make future predictions about potential environmental threats.

The effects of glucocorticoids are mediated by mineralocorticoid receptors (MR) and glucocorticoid receptors (GR), ligand-activated transcription factors that reside mainly in the cytoplasm as part of a multimeric complex when unbound (Funder, 1997). Upon binding with glucocorticoids, the receptor translocates to the nucleus and forms a homodimer that binds to glucocorticoid response elements (GREs) in the promoter or intragenic region of GR-/MR-regulated genes, causing transactivation (reviewed in Ratman et al., 2013). Corticosteroid receptors can also cause transrepression by binding to a negative GRE or can alter gene expression at composite GREs or by tethering to other transcription factors (Ou et al., 2001; Biddie et al., 2011). The pleiotropic effect of corticosteroid receptors stems from the existence of multiple receptor isoforms, subtle differences in GRE sequences, cross-talk with other transcription factors, and the recruitment of various co-factors, to name a few (De Bosscher et al., 2008; Meijsing et al., 2009). GR and MR are also differentially expressed within the brain (MR mainly in limbic regions, GR more widespread) and possess different affinities for

corticosterone (5–10× higher affinity for MR than for GR) (Reul and De Kloet, 1985). Thus, GR binding is more sensitive to fluctuations in circulating concentrations of glucocorticoids, which leads to the hypothesis that MR predominantly affect basal function (e.g., maintain neuronal excitability, cardiovascular function, and circadian rhythm) and GR predominantly mediate stress effects (e.g., altered immune responses, negative feedback) (Gunnar & Quevedo, 2007).

Sex differences in HPA function

There are marked sex differences in HPA activity in rats. For example, basal titers of glucocorticoids are greater in females than males, and their comparable ACTH concentrations suggest that females have enhanced adrenal sensitivity (Atkinson and Waddell, 1997; Babb et al., 2013; Kitay, 1961; Seale et al., 2004a). In addition, relative to males, females have greater concentrations of ACTH and glucocorticoids after acute stress exposure and a slower return to baseline stress levels (Babb et al., 2013; Handa et al., 1994a; Iwasaki-Sekino et al., 2009; Seale et al., 2004a; Viau et al., 2005). Sex differences in PVN activation may underlie the greater stress-induced release of ACTH in females, as studies have shown that expression of the immediate early gene c-fos (marker of neural activation) is upregulated more in females than in males (Babb et al., 2013; Larkin et al., 2010), although others have reported no effect of sex (Viau et al., 2005) or a difference in the other direction (Sterrenburg et al., 2012; Zavala et al., 2011), variability that may be related to the time-points investigated after stress exposures.

Sex-differences in ACTH release may also be mediated by differential expression of HPA-related genes in response to acute stress, with males typically expressing less CRF and AVP messenger RNA (mRNA) in the PVN and less of the ACTH precursor,

proopiomelanocortin (POMC), in the anterior pituitary compared with expression in females (Babb et al., 2013; Iwasaki-Sekino et al., 2009; Seale et al., 2004a; Viau et al., 2005). Taken together, there is evidence of sex differences at each level of the HPA axis, with females typically showing a heightened neuroendocrine response. Nevertheless, females may be partially buffered from higher concentrations of glucocorticoids by their greater concentrations than males of corticosteroid binding globulin (CBG) in circulation (Gala and Westphal, 1965; McCormick et al., 2002), which limit the access of corticosterone to MR and GR (Henley and Lightman, 2011).

Gonadal hormones modulate HPA function

Sex differences in HPA function in adulthood involve, in part, activational effects of gonadal hormones (i.e., modulatory effects that depend on the concentrations of hormone in circulation). The majority of evidence derived from gonadectomy (GDX) and hormone replacement studies indicates that estrogens increase and androgens decrease HPA function (reviewed in Goel et al., 2014). For example, in females, GDX decreases post-stress concentrations of ACTH and corticosterone and causes a quicker return to basal stress levels, and treatment of estradiol reverses the dampening effects of GDX (Burgess and Handa, 1992; Kalil et al., 2013; Leśniewska et al., 1990; Lunga and Herbert, 2004; McCormick et al., 2002; Seale et al., 2004a,b; Viau and Meaney, 1991; Weiser and Handa, 2009). The stimulatory effects of estradiol are not always found, however, (see Babb et al., 2013; Larkin et al., 2010; Young et al., 2001), which may relate to duration and dosage of replacement. In males, GDX increases post-stress concentrations of ACTH and corticosterone and slows the return to basal titers, and androgen treatment typically reverses the effects of GDX (Handa et al., 1994b;

McCormick et al., 1998; Seale et al., 2004a, 2004b; Viau & Meaney, 1996, 2004; Viau et al., 2003).

Effects of gonadal hormones are found at all levels of the HPA axis. For example, estradiol increases adrenal corticosterone content and sensitivity to ACTH, whereas androgens typically exert a dampening effect, contributing to the female-bias in basal and post-stress concentrations of corticosterone (Ajdžanović et al., 2015; Figueiredo et al., 2007; Kitay, 1963; Leśniewska et al., 1990; Funder, 1997; Malendowicz and Mlynarczyk, 1982; Nowak et al., 1995). In the anterior pituitary, POMC mRNA was down-regulated by androgens in males and up-regulated by estradiol in females compared with GDX animals of the same sex when tested after an immune challenge (Seale et al., 2004b). Nevertheless, the effects of gonadal hormones on pituitary ACTH content are conflicting; some studies (Ajdžanović et al., 2015; Critchlow et al., 1963; Kitay, 1963), but not all (Leśniewska et al., 1990; Viau et al., 1999), have found androgens to decrease and estradiol to increase ACTH content. Gonadal hormones may modulate negative feedback in the pituitary, as estradiol reduced GR and MR binding and mRNA expression (Burgess and Handa, 1993; Peiffer and Barden, 1987; Turner, 1990) and, in males, androgens increased nuclear uptake of [^{3}H] corticosterone, in vitro (Viau and Meaney, 2004), although evidence suggests that androgens do not affect MR and GR concentrations or binding, or pituitary sensitivity to CRF (Handa et al., 1994b; Viau and Meaney, 1996).

The effects of estradiol on HPA-related gene and protein expression in the PVN are inconsistent in the literature. For example, estradiol increased stress-induced expression of c-fos mRNA in the PVN of males (Lund et al., 2004a, 2006), but in females

both excitatory (Larkin et al., 2010; Weiser and Handa, 2009) and inhibitory effects (Dayas et al., 2000; Figueiredo et al., 2007; Ter Horst et al., 2009) on c-fos expression have been reported. Further, estradiol augmented the stress-induced increase in CRF heteronuclear (hn) RNA in the PVN of males (Lund et al., 2004a), and, in females, estradiol increased basal and post-stress levels of CRF and AVP mRNA in some studies (Patchev et al., 1995; Seale et al., 2004b); others found no effect of gonadal hormones on one or more of these measures (Figueiredo et al., 2007; Lunga and Herbert, 2004; Paulmyer-Lacroix et al., 1996). There is some evidence, however, that estradiol may modulate CRF expression only during times of greater stimulation of the HPA axis by stressors (Goel et al., 2014; Lund et al., 2004a; Paulmyer-Lacroix et al., 1996).

Two estrogen receptor isoforms, ERα and ERβ, were found to have opposing actions on HPA function (reviewed in Handa et al., 2008). Pylprazoletriol (PPT; binds with greater affinity to ERα than to ERβ) administered into the PVN mimicked the potentiating effects of estradiol on post-stress ACTH and corticosterone and on c-fos expression in the PVN in male and female rats, whereas an ERβ-selective agonist, diarylpropionitrile (DPN), had dampening effects (Liu et al., 2012; Lund et al., 2006; Weiser and Handa, 2009). Gene and protein expression of ERβ is found in the PVN, including the neuroendocrine cells of the medial parvocellular portion (~5–15% co-localize with CRF expression) (Laflamme et al., 1998; Shughrue and Merchenthaler, 2001; Suzuki and Handa, 2005) and ERβ activation was found to influence CRF promoter activity (Miller et al., 2004), leading to the possibility of direct effects on the PVN (Handa and Weiser, 2014). In contrast, the PVN lacks ERα (Laflamme et al., 1998; Miller et al., 2004; Suzuki and Handa, 2005), thus, the ability of estradiol and PPT to

dampen negative feedback is likely mediated by actions on GABAergic neurons of the peri-PVN (Weiser and Handa, 2009). Actions of estradiol likely also occur upstream of the PVN in regions such as the bed nucleus of the stria terminalis (BST), medial preoptic area (MPOA), hippocampus, and amygdala, all of which express ERα and ERβ (Handa and Weiser, 2014).

Androgens typically dampen PVN function in response to stress. In males, stress-induced Fos expression was inversely correlated with plasma testosterone, and GDX increased and prolonged PVN activation (Viau et al., 2003). GDX had no effect on baseline expression of CRF and AVP hnRNA in the PVN of males, but their expression was increased after restraint stress, and AVP hnRNA was negatively correlated with plasma testosterone. Similar effects of GDX have been obtained for the expression of CRF and AVP mRNA after LPS administration, all of which was reversed with either testosterone or dihydrotestosterone (DHT; a more potent, non-aromatizable androgen) (Seale et al., 2004a, 2004b). DHT also reduced expression of c-fos mRNA and CRF and AVP hnRNA after exposure to restraint stress (Lund et al., 2004a, 2006) and recent research indicates that the dampening effects of testosterone implanted near the PVN requires the conversion of testosterone to DHT via 5α-reductase (Handa et al., 2013).

Actions of androgens on PVN function likely occur outside of the PVN because its neuroendocrine cells are lacking in androgen receptors (AR) (Bingham et al., 2006; Handa and Weiser, 2014). The MPOA appears to be an important site for androgen's effects; it is rich in AR, has functional connections to the PVN, and androgen replacement limited to the MPOA decreased AVP hnRNA in the PVN (Williamson et al., 2010) and dampened corticosterone release (McCormick et al., 2002), whereas lesions of

the MPOA prevented the dampening effect (Viau and Meaney, 1996). Further, testosterone facilitated GR binding in the MPOA, but not in other stress-responsive regions (e.g., hypothalamus, pituitary, hippocampus, amygdala) (Viau and Meaney, 1996). The effects of androgens in the posterior BST, however, seem to be opposite to that of the MPOA, as DHT increased Fos-ir and AVP mRNA in the PVN, effects that were blocked with co-administration of an AR antagonist (Bingham et al., 2011). The use of DHT and AR antagonists suggested that ARs mediated the suppressing effect of androgens; more recent work has demonstrated an alternative receptor, which would help to explain the effects of androgens administered directly to the PVN (Handa and Weiser, 2014). Although central actions may require conversion of testosterone to DHT, which too acts at AR, DHT can also be metabolized to 3β-diol, which can bind and activate ERβ, and thereby dampen stress-induced HPA activity (Lund et al., 2004b, 2006; Handa et al., 2011).

Gonadal hormones in early life organize the HPA axis

The extent of influence of the HPG axis on the HPA axis is determined in part by gonadal hormones in early-life (i.e., organizational/programming effects). Although gonadal function is relatively quiescent until puberty, males are exposed to two surges in testosterone release, one late in gestation and one soon after birth. Neonatal GDX or antagonism of testosterone's actions at AR reduced male rats' sensitivity to testosterone's dampening effects on corticosterone release in response to stress in adulthood (Bingham and Viau, 2008; McCormick et al., 1998). Females given a single dose of testosterone as neonates also exhibited a dampened stress response as adults (Seale et al., 2005a). Testosterone's organizational effects in the neonatal period involve both AR and ER

(through testosterone's conversion to estradiol by aromatase); males treated with either an AR antagonist or an aromatase inhibitor during the perinatal period had increased basal and stress-induced concentrations of corticosterone in adulthood (Bingham et al., 2011, 2012; McCormick and Mahoney, 1999; Seale et al. 2005b).

In males, neonatal GDX increased MR binding and decreased GR binding in the adult anterior pituitary (McCormick et al., 1998) and both antagonism of AR and inhibition of aromatase reduced GR mRNA expression in the PVN (Seale et al., 2005b). In females, neonatal treatment of testosterone or estradiol increased adult expression of GR mRNA in the PVN (Patchev et al., 1995; Seale et al., 2005a). In the adult hippocampus, GR mRNA expression was lower in females administered estradiol as neonates (Patchev et al., 1995), although adult corticosteroid receptor mRNA expression in the hippocampus was unaffected by neonatal blockade of AR or aromatase inhibition in males (Seale et al., 2005b). Together, these findings suggest that perinatal androgen exposure and actions at AR and ER may enhance HPA negative feedback in adulthood in a region-specific manner.

Androgen exposure in neonatal life reduced HPA drive in adulthood as evidenced by reduced neural activation in the PVN in response to stress (Bingham and Viau, 2008), and neonatal treatment with an aromatase inhibitor increased neural activation in various stress-responsive brain regions (e.g., PVN, lateral septum, amygdala) (Bingham et al., 2012). Gene expression of CRF and AVP in the PVN as well as POMC in the anterior pituitary is also up-regulated in males given AR antagonists or aromatase inhibitors perinatally, whereas females given testosterone perinatally had down-regulated expression after stress in adulthood (Seale et al., 2005a, 2005b). In contrast, adult females

treated with estradiol as neonates had up-regulated CRF and AVP mRNA in the PVN (Patchev et al., 1995). Bingham and Viau (2008) found no effect of neonatal testosterone exposure on adult CRF and AVP mRNA in the PVN, but did see increased expression of AVP and AR in the BST and medial amygdala, providing further evidence that early androgen exposure in early-life modulates adult sensitivity to androgens. Moreover, the organizational effects of testosterone on AVP in the BST and medial amygdala are at least in part related to ER activation (Bingham et al., 2012; Han and De Vries, 2003). The divergent findings regarding adult expression of HPA-related genes in the PVN may be related to differences in the timing and duration of treatment in early-life. Nevertheless, the available evidence indicates that adult HPA function is shaped by perinatal gonadal hormones.

Adolescence as a transitional period in HPG–HPA interactions

Although there is no clear onset or offset, adolescence is broadly defined in rodents as postnatal day (P) 22 to P59, with markers of puberty occurring at about P35 in females and P42 in males, and P60 considered young adulthood (reviewed in McCormick and Green, 2013). Adolescence is characterized by marked shifts in behaviour, such as increased risk-taking, greater social interaction, and increased propensity for drugs of abuse (Doremus-Fitzwater et al., 2010). While some of these behaviours may be detrimental (e.g., greater sensitivity to alcohol) and a consequence of adaptive maturational processes, most are seen to prepare the organism for adult-life by promoting independence. Changes in behaviour reflect pronounced shifts in neuroendocrine function (e.g., HPG maturation associated with puberty) and neurobiology (e.g., changes in white matter, receptor densities, synaptic pruning, and neurogenesis) (Casey et al., 2008; He

and Crew, 2007; Varlinskaya et al., 2013). Developmental periods of heightened brain maturation (e.g., adolescence), may render the brain vulnerable to environmental influences. For example, the consequences of chronic stress are more long-lasting and detrimental when experienced in adolescence compared with in adulthood (Green and McCormick, 2013; McCormick and Green, 2013). Moreover, developmental changes in HPA activity during adolescence could be a contributing factor and thus, investigations into developmentally-related differences in HPA reactivity and downstream effects of stress signaling are important for elucidating markers of vulnerability or resilience.

Basal titers of glucocorticoids increase until approximately P45, then stabilize at adult-typical levels (Pignatelli et al., 2006). Adrenal volume increases until ~P60, but more so in females than in males (Pignatelli et al., 2006). In response to a variety of stressors, prepubertal rats of both sexes typically exhibit a more pronounced and slower to recover ACTH and corticosterone response compared to adults (Goldman et al., 1973; Hall and Romeo, 2013; Lui et al., 2012; Romeo et al., 2004a,b, 2006a,b; Vásquez and Akil, 1993). Greater HPA reactivity before puberty is likely attributable to a combination of factors such as maturing adrenal and pituitary function (Foilb et al., 2011; Lui et al., 2012; Romeo et al., 2004b), greater stress-induced PVN activation (Lui et al., 2012; Romeo et al., 2006a; Viau et al., 2005), and developmental shifts in signaling upstream of the PVN (Weathington and Cooke, 2012). The prolonged corticosterone release of prepubertal rats may involve reduced negative feedback (Goldman et al., 1973; Vásquez and Akil, 1993), and some studies have found gene expression of corticosteroid receptors in the hippocampus to increase between P30 and adulthood (Bohn et al., 1994). Nevertheless, others have found GR expression in stress-related brain regions to remain

stable (Dziedzic et al., 2014) or to decrease in adolescence (Meaney et al., 1985). Further, we have found comparable GR expression at P35, P45, and P75, but greater GR translocation in response to stress in adolescents than in adults (unpublished data)[1]; less translocation would be more consistent with reduced negative feedback.

Adult-like function of the HPA axis emerges at ~P60 (Foilb et al., 2011; Gomez et al., 2002). The developmental shift in HPA function is not explained by the low gonadal function in pre-pubertal adolescents relative to adults. When prepubertal males were GDX and provided adult titers of testosterone, they continued to display a protracted corticosterone response to restraint compared with similarly treated adults(Romeo et al., 2004a). AR antagonism had no effect on HPA function in P40 rats, but increased stress-induced ACTH release in adults (Gomez et al., 2004). HPA–HPG interactions change during adolescence in females as well; corticosterone remained elevated for longer after restraint in GDX prepubertal rats compared with GDX adult rats (Romeo et al., 2004b). Furthermore, estradiol treatment reduced the ACTH and corticosterone response to stress in prepubertal rats, in contrast to the enhancing effects observed in adulthood (Evuarherhe et al., 2009b).

Outstanding questions for understanding HPG–HPA interactions in adolescence

It is unclear why the HPA axis does not respond (in males) or responds differently (in females) to gonadal hormones before puberty. Androgens and estrogens are capable of modulating the expression of gonadal receptors and aromatase before puberty, as in adults (Lephart and Ojeda, 1990; Romeo et al., 2002). In the anterior hypothalamus, mRNA expression of AR, ERα, ERβ, and progesterone receptors (PR) is similar at P30

[1] At the time that this review chapter was submitted for publication, the data were unpublished. The data have since been published and are included in the article that comprises Chapter 2 of this thesis.

and P60 (Walker et al., 2009), although age-related changes may exist in other regions; in the PVN of males, fewer cells express PR before puberty than in adulthood (Romeo et al., 2005). It remains unclear, however, how progesterone activity modulates HPA function during the adolescent period, which is an important question considering that progesterone dampens HPA function in adulthood and that prepubertal rats secrete more progesterone in response to stress compared to adults (Romeo et al., 2005; Viau and Meaney, 1991). Evidence from our lab indicates that, in males, the progesterone response to stress does not mature until after puberty, as both P35 and P45 rats had a markedly greater response compared to young adults (unpublished data)[2].

Puberty-related shifts in HPA function may involve differential binding of hormones and their metabolites to gonadal receptors as well as to shifts in receptor function. Aromatase and 5α-reductase expression in the brain decreased in mid to late adolescence until reaching adult-typical levels (Ivanova and Beyer, 2000; Lephart et al., 2001). Thus, treatment with gonadal hormones may result in differential conversion to various metabolites before puberty compared with in adulthood. Another possibility is that age-related differences in the effects of gonadal hormones are mediated by shifts in receptor chaperones and co-factors. For example, nuclear receptor activity modulates the expression of a variety of co-chaperones (e.g. FK506 binding protein 51 [Fkbp5] and peptidylprolyl isomerase D [Ppid]) that in turn modulate nuclear receptor activity (reviewed in Binder, 2009; Malviya et al., 2013). Nevertheless, whether the expression of proteins that modulate nuclear receptor activity changes in adolescence and whether gonadal hormones regulate the expression of these proteins in adolescence the way they do in adulthood is unknown. Age-difference in HPG–HPA interactions may also involve

[2] At the time that this review chapter was submitted for publication, the data were unpublished. The data have since been published and are included in the article that comprises Chapter 2 of this thesis.

developmental shifts in co-factors that modulate the transcriptional effects of nuclear receptors; in mice, the expression of steroid receptor co-activators (SRC) changes in the hippocampus from P18 to adulthood (Schmidt et al., 2007) and in female rats, hypothalamic expression of SRC-1 changed throughout adolescence, but its gene expression was greatest at puberty (P40) (Mitev et al., 2003).

A fundamental question to be answered is when in adolescence does the HPA axis become sensitive to regulation by the HPG axis. Investigations in postpubertal animals are lacking. We have evidence that the HPA axis of postpubertal adolescents (P45), in contrast to prepubertal adolescents (P35), is indeed sensitive to testosterone, but in adirection opposite to that of adults. Testosterone replacement increased, rather than decreased, corticosterone release in response to restraint stress in GDXed P45 males (unpublished observations)[3]. This effect may be related to developmental changes in enzymes that convert testosterone to estradiol and/or convert DHT to 3β-diol.

Although there is limited evidence for HPG regulation of the HPA axis in prepubertal adolescence, there is some evidence that adolescence may be a second window for organizational effects of hormones on the HPA axis. Adult males were unresponsive to the dampening effects of testosterone treatment on corticosterone concentrations and on CRF and AVP mRNA in the PVN, if they were GDXed as prepubertal adolescents (Evuarherhe et al., 2009a). Prepubertal GDX, however, did not alter AR or ER expression in various HPA-related brain regions (Brown et al., 2015). In adolescence, testosterone altered the ongoing development of regions implicated in HPA activity (e.g., amygdala, MPOA) (reviewed in Schulz et al., 2009; Cooke and Shukla,

[3] At the time that this review chapter was submitted for publication, the data were unpublished. The data have since been published and are included in the article that comprises Chapter 3 of this thesis.

2011). Nevertheless, some of the differences between adolescents and adults in HPA function may involve ongoing maturation of these structures.

Lastly, evidence of a different regulation of the HPA axis by the HPG axis in adolescence than in adulthood begs the question of the functional significance of these changes. For example, there is a stress hyporesponsive period from approximately P2 to P12 in which many stressors are unable to activate the HPA axis, which has been postulated to protect ongoing brain development from elevations in glucocorticoids (Sapolsky and Meaney, 1986). The shifts in HPG regulation of the HPA axis may be relevant for other significant differences between adolescents and adults. Adolescence is a time of social restructuring, a critical period for social learning, and a time when social interactions have higher reward relative to in adulthood (reviewed in Doremus-Fitzwater et al., 2010). Is the differential function of the axes essential for the expression of the behavioral repertoire that is unique to the adolescent period of life? Do the shifts facilitate the secondary phase of sexual differentiation that occurs in adolescence? Understanding of HPG–HPA interactions in adolescence may be critical to understanding the heightened susceptibility of adolescents to the detrimental consequences of chronic stressors relative to adults (McCormick and Green, 2013; Romeo, 2013).

Goals of the thesis[4]

The above review of the literature provides the rationale for the questions addressed in my thesis, the overarching goal of which was to characterize and investigate the mechanisms underlying developmental shifts in HPA function and its regulation by gonadal hormones during adolescence and early adulthood. In Chapter 2, I characterized the hormonal profile (corticosterone, progesterone, testosterone) of male rats at baseline

[4]This section is not found in the published version of this Chapter, but has been included to provide a general overview of the objectives of this thesis.

and in response to acute stress before, during, and after puberty. I then investigated whether age-related differences in stress responsivity involved changes in GR translocation and signaling in the hippocampus, by measuring GR expression in the cytosol and in the nucleus and gene expression of corticosteroid receptors and their co-chaperones before and after stress. I provide novel evidence that age-related differences in the neuroendocrine function are unrelated to GR activity in the hippocampus and advance our general understanding of HPA maturation throughout adolescence.

In Chapter 3, I examined whether developmental shifts in HPA function are related to changes in circulating gonadal hormones, which would provide a parsimonious explanation for age-related differences; in adult males, testosterone dampens HPA function and facilitates a return to baseline titers of corticosterone (Goel et al., 2014). Although a previous study found that age-related differences persisted when pre-pubertal adolescent males were given testosterone (Romeo et al., 2004a), the study involved small group sizes (n = 5 per group). Moreover, if testosterone could not regulate HPA responses prior to puberty, as reported by Romeo and colleagues (2004a), then it begs the question of how and when does HPA function become sensitive to gonadal regulation. Therefore, I examined the effects of testosterone on baseline and post-stress measures of HPA function (circulating corticosterone and PVN expression of Fos and AVP) at different ages. In general, I found evidence for developmental shifts in HPA function and in its regulation by gonadal hormones, which I hypothesized were related to differential conversion of testosterone to its metabolites based on previous evidence for developmental changes in central expression of aromatase and 5α-reductase between adolescence and adulthood (Lephart & Ojeda 1990). Because estradiol can increase stress

via ERα, and because DHT can dampen stress via AR or be metabolized into 3β-diol to have dampening effects via ERβ, I compared the effects of AR and ER antagonists to vehicle in OCX males treated with testosterone or DHT on post-stress release of corticosterone and progesterone. Together, these finding provide evidence that developmental shifts in HPA function are independent of gonadal status, and that gonadal regulation of HPA function changes during adolescence in a marker-specific manner.

In Chapter 4, I investigated ovarian regulation of HPA function in adulthood. Previous research indicates that estradiol potentiates HPA responses and reduces negative feedback in both males and females, but the findings have not always been consistent. Moreover, evidence from cell culture studies indicates that estradiol's actions may involve dampening of GR activity (Malviya et al., 2013). Therefore, I investigated the effects of OVX and of ovarian hormone replacement (estradiol alone or in combination with progesterone) in adulthood on corticosterone responses and hippocampal GR translocation and expression of certain genes of interest, which code for proteins involved in altering GR assembly, affinity, translocation, or transcriptional properties. The findings generated from this study clarify the effects of gonadal regulation of HPA function and provide evidence for underlying mechanisms mediating their effect. As such, these findings provide potential targets for future investigations into developmental changes in gonadal regulation of HPA function during adolescence in females. The results of these studies may also elucidate mechanisms that underscore the development of stress-related disorders (e.g., depression and anxiety), which typically emerge during adolescence in a sex-specific manner.

Figures

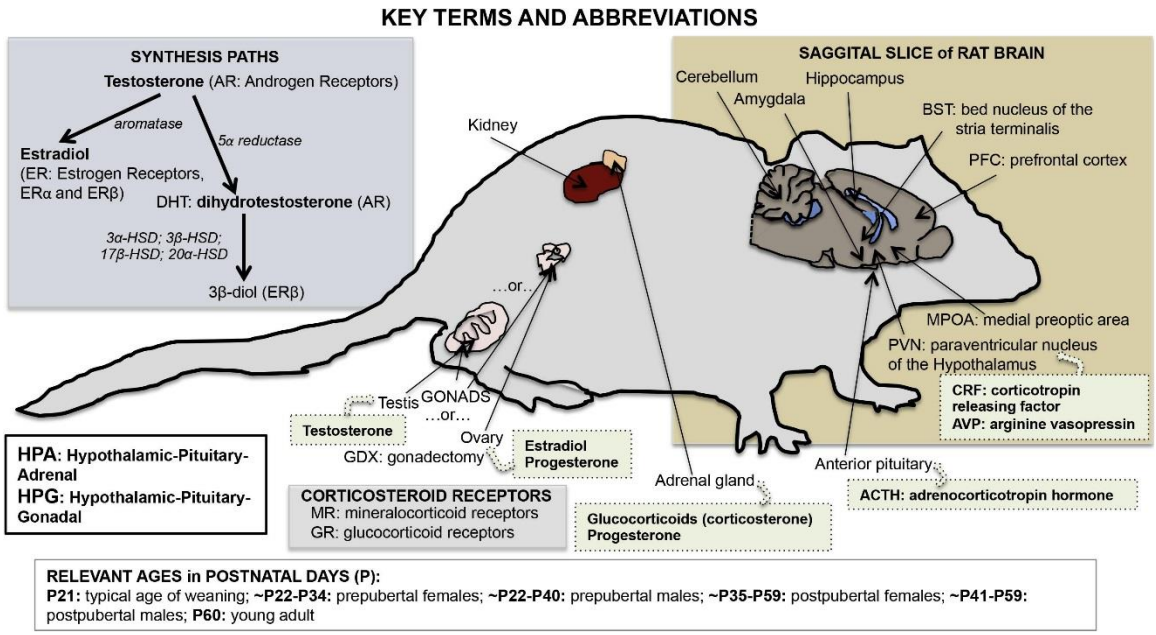

Fig. 1-1. The figure includes a cartoon outline of a rat depicting relevant structures, a chart of the synthesis pathways of testosterone to estradiol and dihydrotestosterone (receptors for each metabolite are within parentheses; enzymes are italicized), key terms, and abbreviations used in the review (see Handa and Weiser, 2014 for discussion of the synthesis pathways).

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Rationale for Chapter 2

In this chapter, I investigated the mechanisms underlying developmental shifts in HPA function. To do so, I first characterized the hormonal profile (corticosterone, progesterone, testosterone) of pre-pubertal and (P35) post-pubertal (P45) adolescent and adult (P75) male rats at baseline and in response to an acute stressor. I included post-pubertal adolescents (P45), for which there are fewer investigations, to better understand the transition into adulthood in an age group that is undergoing marked hormonal changes. After I demonstrated developmental shifts in the hormonal profiles of male rats, I investigated GR signaling as a basis for age-related differences in HPA function, because previous research has provided evidenced for impaired negative feedback. To do so, I measured GR expression in the cytosolic and nuclear compartment of hippocampal tissue before and after acute restraint stress. I predicted that pre-pubertal males would have reduced translocation of GR from the cytosol to the nucleus in response to restraint stress. I also investigated age-related differences in mRNA expression corticosteroid receptors and their co-chaperones that modulate GR receptor assembly, binding affinity, and translocation as potential sources for differential GR activity.

Chapter 2: Glucocorticoid receptor translocation and expression of relevant genes in the hippocampus of adolescent and adult male rats.

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Author contribution: I was the primary investigator of this work, responsible for the majority of study design, data collection, data analysis, and writing of the manuscript. I performed hormone assays, Western blot experiments, and assisted in primer design, RNA extraction, and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) experiments.

Rachel Nottrodt helped design primers (50%), extract RNA (50%), and performed reactions for RT-qPCR (100%). Jonathan Simone assisted with sample collection (20%), the development of the fractionation protocol (10%), and editing the manuscript. Dr. Cheryl McCormick assisted in designing the experiments (30%), analyzing the data (20%), and editing the manuscript.

Introduction

The release of glucocorticoids (primarily corticosterone in rodents) is regulated by the hypothalamic-pituitary-adrenal (HPA) axis, which help individuals to meet the challenges of their environment by modulating glucose metabolism, cardiovascular function, immune function, and psychological processes (Sapolsky et al., 2000; Groeneweg et al., 2011). Actions of glucocorticoids are mediated predominantly by two intracellular receptors (mineralocorticoid and glucocorticoid receptors; MR and GR), which act as transcriptional regulators and differ in their distribution in the brain and in their affinities for glucocorticoids; at basal concentrations, unlike MR, GR are mostly unoccupied (Reul and de Kloet, 1985). Therefore, GR are more sensitive to stressor-induced increases in glucocorticoid concentrations and are believed to mediate HPA feedback, whereby elevations in circulating glucocorticoid concentrations suppress further HPA activation, preventing over-exposure (Groeneweg et al., 2011; Herman et al., 2012).

Adolescence is a transitional stage of development that involves significant changes in the functioning of the HPA axis. In rodents, it is well-documented that pre-pubertal adolescents have greater and more protracted corticosterone responses to stressors (e.g., restraint, ether exposure, foot shock) compared with responses found in early life (P1–14 days of age) and in adulthood (>P60 days of age) (Klein and Romeo, 2013; Green and McCormick, 2016). The pronounced release of corticosterone in pre-pubertal adolescent rats may be related to ongoing maturation at any level of the HPA axis. For example, the adrenals of pre-pubertal adolescents weigh more than those of adults after normalizing to total body weight, release more corticosterone in response to stressors, and differ in their response to exogenous adrenocorticotropic hormone (ACTH)

(Romeo et al., 2004a, 2005, 2014; Romeo, 2010; Foilb et al., 2011; Dziedzic et al., 2014; Hall and Romeo, 2014). Further, after exposure to a stressor, pre-pubertal rats have greater circulating concentrations of ACTH compared with adults (Romeo et al., 2006b; Foilb et al., 2011; Dziedzic et al., 2014; Hall and Romeo, 2014) and greater and/or more prolonged activation of the paraventricular nucleus (PVN) of the hypothalamus, as evidenced by increased expression of immediate early genes and their protein products (e.g., Fos and Egr-1) relative to adults (Viau et al., 2005; Lui et al., 2012; Hodges et al., 2014), particularly in cells that are immunoreactive for corticotropin releasing factor (CRF) (Romeo et al., 2006a). There are also differences in gene expression of CRF and arginine vasopressin (AVP) in the PVN of pre-pubertal and adult rats, although the findings are somewhat inconsistent (Viau et al., 2005; Romeo et al., 2007).

The exaggerated HPA reactivity that is evident in pre-pubertal males likely is unrelated to developmental changes in hypothalamic-pituitary-gonadal (HPG) function (onset of puberty occurs approximately at postnatal day [P] 34 in female rats and P42 in males) and in circulating gonadal hormones, which are known to affect the stress response in adults; estradiol increases and testosterone decreases HPA reactivity (Goel et al., 2014; Green and McCormick, 2016). Pre-pubertal males continued to show a greater and more prolonged release of ACTH and corticosterone compared with adults when males of both ages were gonadectomized and administered testosterone to mimic adult-typical concentrations (Romeo et al., 2004a). The difference between pre-pubertal and adult females also persisted when both age groups were ovariectomized, suggesting that age-related differences in HPA function are not mediated by changes in ovarian hormones (Romeo et al., 2004b). Lastly, although post-pubertal adolescents (>∼P42

and <P60) have not been investigated to the same extent as pre-pubertal adolescents, the available evidence suggests that HPA function is still maturing during this time and into late adolescence (~P50–P60) (Foilb et al., 2011; Hodges and McCormick, 2015).

The exaggerated HPA reactivity and slower recovery in pre-pubertal males may be because HPA negative feedback is immature; administration of cortisol or the GR agonist dexamethasone was less effective at suppressing the hormonal response to stressors in pre-pubertal adolescents compared with in adults, which suggests ongoing maturation of HPA feedback during adolescence (Goldman et al., 1973; Vazquez and Akil, 1993). No differences were found, however, between adolescents and adults, in GR protein and gene expression in regions involved in HPA negative feedback (e.g., PVN, pituitary, hippocampus, and medial prefrontal cortex) in rodents (Vazquez, 1998; Romeo et al., 2008, 2013; Dziedzic et al., 2014). Nevertheless, there may be age-related differences in the affinity or activity of GR that cannot be seen by measuring overall expression.

A related possibility is that changes during the adolescent period in the expression of co-chaperones involved in modulating GR activity are involved in age-related differences in HPA function. For example, FK506 binding protein 51 (Fkbp51, encoded by the *FKBP5* gene) acts as a suppressor of GR activity by inhibiting the receptor's ability to bind its ligand and translocate to the nucleus to affect gene expression (Binder, 2009). Bag1 is thought to inhibit GR activity by interfering with the receptor's assembly, by reducing receptor translocation, and by binding to the receptor's hinge region and impeding DNA binding (Schmidt et al., 2003). In contrast, Fkbp52 (encoded by the *FKBP4* gene) can be exchanged for Fkbp51 in the GR complex, and promotes

translocation into the nuclear compartment by recruiting the motor protein dynein (Davies et al., 2002). Furthermore many of the hormones that regulate chaperone expression (Hubler and Scammell, 2004; Binder, 2009; Malviya et al., 2013) increase in concentration during adolescence.

In Experiment 1, we investigated age and stressor effects on hormone concentrations (corticosterone, testosterone, and progesterone) and on GR translocation in the hippocampus, a region that richly expresses corticosteroid receptors and is involved in negative feedback (Reul and de Kloet, 1985; Herman and Cullinan, 1997). Based on previous findings, we predicted that pre-pubertal adolescent males would have greater corticosterone and progesterone release in response to an acute stressor than would adults (Romeo et al., 2005, 2006b). Because pre-pubertal rats are postulated to have dampened feedback relative to adults (Goldman et al., 1973; Vazquez and Akil, 1993), we also tested the hypothesis that prolonged corticosterone release in pre-pubertal adolescent rats involved reduced GR translocation from the cytoplasm to the nucleus after restraint stress. In Experiment 2 we investigated developmental and stressor-induced changes in the expression of genes that code for corticosteroid receptors and their chaperone proteins within the hippocampus (i.e., *NR3C1, NR3C2, FKBP5, FKBP4, BAG1*). In addition to investigating pre-pubertal adolescents (P35) and adults (P75), we investigated post-pubertal adolescents (P45) to gain insight into an age group for which less is known about HPA function to better understand the transition into adulthood.

Methods

Experiment 1

Animals

For Experiment 1a, male Long-Evans rats were obtained at P30 (*n* = 32; 80–115 g), P40 (*n* = 32; 180–220 grams), and P70 (*n* = 32; 300–385 g) from Charles River (St. Constant, Quebec) and housed in same-aged pairs. For Experiment 1b, a second batch of males arrived at P30 (*n* = 16; 100–130 g), P40 (*n* = 16; 175–215 g), and P70 (*n* = 16; 300–355 g). Animals were given free access to food and water and kept on a 12 h light-dark cycle (lights on at 09:00). All procedures were approved by the Brock University Animal Care and Use Committee and were in keeping with the National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) as well as the Canadian Council on Animal Care guidelines.

Acute stressor procedure and sample collection

The acute stressor procedure and sample collection began four to six days after the animals arrived at the facility, and thus were (±1 day) P35, P45, or P75 at collection. On each collection day, all age groups and time-points were represented. Within ~2–5 h after lights on, rats were decapitated either directly from the home cage (baseline) or immediately after 30 min of restraint stress (post-restraint) in Plexiglas® restrainers (Experiment 1a), or after 30 min of recovery after the restraint stress (Experiment 1b), for the collection of trunk blood and brains. This range in time of day was chosen for the experiments so that samples were collected during the phase of the light cycle when basal plasma corticosterone concentrations and the percentage of bound GR are low; these conditions would better allow investigation of the effects of stress on GR translocation. Trunk blood was collected into ice-chilled glass tubes containing EDTA and centrifuged at 3000 RCF for 15 min. Plasma was collected and stored at −20° C until hormone assays

were conducted. Brains were quickly extracted, sliced into 1 mm thick sections on ice, and frozen on dry ice before stored at −80° C until further processed.

Hormone assays

Steroids were extracted from plasma using diethyl ether for the measurement of corticosterone, testosterone, and progesterone. All extracted samples were reconstituted in buffer provided in the enzyme-linked immunosorbent assay kits (Neogen, Lansing MI) and the assays were conducted according to the kit instructions and using a Biotech Synergy plate reader; all samples within an experiment were measured on the same day. Assay sensitivity was 0.05 ng/mL for corticosterone, 0.002 ng/mL for testosterone, and 0.4 ng/mL for progesterone.

Protein extraction and subcellular fractionation

The whole hippocampus was dissected on dry ice and the tissue from two animals of the same age and time-point were pooled (~100 mg of tissue per sample; n = 8 pooled samples for each age group at each of the baseline and post-restraint time points). Throughout the protein extraction, samples were kept on ice or at 4 °C. An appropriate volume of S1 buffer (10 mM HEPES pH 7.5, 1.5 mM MgCl2, 10 mM KCl, 2 mM DDT, 1 mM EDTA, 1 mM EGTA, 0.5% Triton-X; with protease and phosphatase inhibitors [Roche]) was added to each sample, tissue was homogenized in a Bullet Blender®, and the homogenate was centrifuged (17200 RCF for 3 min), after which the supernatant (SN1) was collected for use as the cytosolic fraction and the pellet was retained for use as the nuclear fraction. SN1 was centrifuged (16000 RCF for 20 min) and the supernatant was collected for use as the final cytosolic fraction. The original nuclear pellet was washed by re-suspending it in S1 buffer using a 25 gauge needle and vortex, and then

was re-centrifuged (17200 RCF for 3 min). The supernatant was discarded and the washed pellet was re-suspended in an appropriate volume of S2 buffer (~1.2 pellet volumes; 20 mM HEPES, 400 mM NaCl, 1.5 mM MgCl2, 0.1 mM EDTA, glycerol, 2 mM DTT; with protease and phosphatase inhibitor [Roche]) using a 25 gauge needle, after which the samples incubated on ice for 1 h, with a vortex every 10 min. After incubation, the samples were centrifuged (17200 RCF for 30 min) and the supernatant was obtained for use as the final nuclear fraction. The amount of protein in the cytosolic and nuclear fractions was determined via the Bio-Rad Protein Assay procedure (Bio-Rad Laboratories). Samples were brought to an equal concentration using the appropriate buffer, and an equal volume of 2x Laemmli buffer was added. Samples were heated at 70 °C for 3 min, centrifuged (16000 RCF for 10 min), and stored at −20 °C until use in Western blot experiments.

Western blot

To measure GR translocation, cytosolic and nuclear samples (30 μg of protein each) were resolved via SDS-PAGE on 7.5% polyacrylamide gels and transferred to a PVDF membrane (Millipore). Total protein was measured via Ponceau S stain and used to normalize GR values. Membranes were reactivated in methanol, washed (3 × 5 mins) in Tris-buffered saline with 1% Tween 20 (TBS-T), and blocked with 5% non-fat milk powder dissolved in TBS-T. Membranes incubated overnight at 4 °C in TBS-T containing 1% milk block and rabbit anti-GR (sc-1004, Santa Cruz; 1:500 and 1:200 for cytosolic and nuclear samples, respectively). The next day, membranes were washed, and then incubated for 45 min at room temperature in TBS-T containing 1% milk block and goat anti-rabbit (Alexa488, Life Technologies; 1:5000), washed again, and then were

imaged using a Bio-Rad VersaDoc (MP 4000). Each membrane included samples from each age and time-point (Experiment 1a) or each age (Experiment 1b).

To validate the subcellular fractionation protocol, cytosolic and nuclear samples (10 μg of protein each) were resolved on 12.5% polyacrylamide gels, blocked with 5% BSA in TBS-T, and probed with rabbit anti-histone H3 (D1H2, Cell Signaling; 1:5000) and rabbit anti-GAPDH (14C10, Cell Signaling; 1:5000) in TBS-T with 1% BSA. The results indicated minimal contamination of the cytosolic and nuclear compartments and validated our fractionation (see Fig. 2-2A).

Experiment 2

Animals

To measure gene expression, male Long Evans rats arrived at P29 (n = 16; 65–90 g), P39 (n = 16; 125–205 g), and P69 (n = 16; 260–350 g). Animals were given free access to food and water and kept on a 12 h light-dark cycle (lights on at 09:00). All procedures were approved by the Brock University Animal Care and Use Committee and were in keeping with the National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) as well as the Canadian Council on Animal Care guidelines.

Acute stressor procedure and collection of brain tissues

On P35, P45, or P75, animals were decapitated ~3–5 h into the light phase either directly from the home-cage (baseline) or 90 min after 30 min of restraint stress (post-restraint). The post-restraint time point was chosen to allow the time for changes in gene expression downstream of GR translocation, and adapted from Bourke et al. (2013). Brains were collected as described in Experiment 1.

RT-qPCR

A total of eight 1 mm inner diameter punches from the dorsal and ventral hippocampus (2 punches per region and per hemisphere in 1 mm thick slices; ~20 mg of tissue per sample) were collected on dry ice and total RNA was extracted using a Fatty Tissue RNA Purification Kit (Norgen, #36200) as per kit instructions. RNA concentration and quality was assessed using a Nanodrop and samples (500 ng of total RNA) were reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, # 1708891). Primers were designed to measure mRNA expression of various stress-related genes (see Table 2-1). Samples were run in triplicate using CFX Connect Real-Time System and SYBR green (Bio-Rad Laboratories) in a 96 well format and samples were counterbalanced within and across plates. Gene expression was normalized to Cyclophilin A and GAPDH, which demonstrated good target stability (mean CV = 0.06; mean M = 0.17) and showed no effect of age, of time-point, or interaction. Efficiencies were between 90 and 110% and no template controls (NTC) and no reverse transcriptase (NRT) controls indicated that there were no issues with primers and with genomic DNA contamination, respectively.

Statistical analyses

Experiments 1A and 2 involved 3 (age: P35, P45, P75) x 2 (time-point: baseline, post-restraint) between-subjects ANOVAs and Experiment 1b involved one-way (age: P35, P45, P75) ANOVAs. Because animals from Experiments 1a and 1b arrived in separate shipments from which samples were collected, dissected, processed, and tested separately, we did not make direct comparisons between the time-points in the two experiments. Alpha was set at $p < 0.05$ and significant interactions were followed-up with

independent samples *t*-tests (to compare the effect of time-point at each age) or with one-way ANOVAs (to examine developmental changes at each time-point) and post hoc comparisons were Bonferroni corrected. For hormone concentrations and GR translocation (Experiments 1a and 1b) data analyses were conducted using SPSS 16. RT-qPCR results (Experiment 2) were computed using the Bio-Rad CFX Manager and analyzed using GraphPad Prism 7.

Results

Experiment 1a

Hormones

Age-related differences in testosterone concentrations depended on time-point ($F_{2,90} = 6.57$, $p = 0.002$). At baseline ($F_{2,45} = 6.98$, $p = 0.002$), males at P35 had lower testosterone than did males at P45 and P75 ($p = 0.003$ and 0.022, respectively), which did not differ ($p = 1.0$). Post-restraint ($F_{2,45} = 14.68$, $p < 0.001$), males at P75 had greater concentrations than did males at P35 and P45 ($p < 0.001$ and $p = 0.002$, respectively), which did not differ ($p = 0.320$). Alternative analyses, performed to examine the effect of time-point on testosterone at each age, indicated that when compared with baseline, post-restraint testosterone was lower in males at P35 ($p = 0.037$), did not differ at P45 ($p = 0.113$), and was higher in males at P75 ($p = 0.031$; see Fig. 2-1A).

For concentrations of progesterone, there was an interaction of age and time-point ($F_{2,81} = 4.24$, $p = 0.018$). At baseline ($F_{2,38} = 3.30$, $p = 0.048$), age-related differences were not significant after correction for pairwise comparisons, although there was a trend for P45 males to have greater concentrations of progesterone than P75 males ($p = 0.080$). Post-restraint ($F_{2,43} = 6.33$, $p = 0.004$), males at P35 and P45 had significantly greater

concentrations of progesterone than did males at P75 (*p*s = 0.008 and 0.012, respectively) and the two younger groups did not differ ($p = 1.0$; see Fig. 2-1B).

The interaction between age and time-point was also significant for corticosterone concentrations ($F_{2,90} = 5.66$, $p = 0.005$). There were no age-related differences in concentrations of corticosterone at baseline ($p = 0.409$). Post-restraint ($F_{2,45} = 5.97$, $p = 0.005$), P35 males did not differ from P45 males ($p = 0.385$), and as predicted P35 males had greater concentrations than P75 males ($p = 0.004$). When one outlier (>−2 SD) in the P45 group was removed, their concentrations of corticosterone post-restraint were greater than P75 males, but the difference did not meet the criteria for statistical significance ($p = 0.051$; see Fig. 2-1C).

Bivariate correlations were performed to examine the relationship of hormones at each age and time-point to see if the correlation found between progesterone and corticosterone in adults after exposure to a stressor (Hueston and Deak, 2014) is evident in adolescents. There were no significant correlations between hormones in rats of any age at baseline (*p*s > 0.05). Post-restraint, however, concentrations of corticosterone and progesterone were positively correlated at all three ages (P35, $r = 0.63$, $p = 0.009$; P45, $r = 0.44$, $p = 0.089$; P75, $r = 0.78$, $p = 0.001$), although the relationship failed to reach statistical significance in P45 males (see Fig. 2-1D).

Glucocorticoid receptor translocation

We measured GR expression in the cytosolic and nuclear fractions of hippocampal tissue collected at baseline and post-restraint. In cytosolic samples, there was no effect of age ($p = 0.900$) and, as predicted, there was an effect of time-point ($F_{1,36} = 5.82$, $p = 0.021$). Expression of GR in the cytosolic compartment was reduced

post-restraint (see Fig. 2-2B and 2-2D). For nuclear samples, there was a main effect of age and of time-point ($p = 0.001$ and $p < 0.001$, respectively), however, we investigated the interaction, which nearly reached statistical significance ($F_{2,37} = 3.22$, $p = 0.051$). There were no age-related differences observed at baseline ($p = 0.125$). Post-restraint ($F_{2,17} = 5.76$, $p = 0.012$), nuclear GR expression was greater in P35 males compared to P75 males ($p = 0.011$; see Fig. 2-2C and 2-2E), whereas expression in P45 males was intermediate and statistically did not differ from their younger or older comparisons (ps = 0.258 and 0.361, respectively).

Experiment 1b

Hormones

Concentrations of testosterone 30 min post-restraint differed based on age ($F_{2,44} = 32.74$, $p < 0.001$). Specifically, males at P75 had greater concentrations of testosterone than did males at P35 and P45 (ps < 0.001), which did not differ ($p = 0.65$; see Fig. 2-3A). For progesterone, there was no longer an effect of age ($p = 0.167$; see Fig. 2-3B) and all groups had returned to approximate baseline concentrations (~1–3 ng/mL) as was seen in Experiment 1a (see Fig. 2-1B). For corticosterone, we predicted that adolescent males would have greater concentrations than adult males based on past findings. Similar to progesterone, however, concentrations of corticosterone had already returned to approximate baseline values (~5–25 ng/mL), although there was an effect of age ($F_{2,44} = 4.18$, $p = 0.022$); P45 males had less circulating corticosterone than did P75 males ($p = 0.018$), which did not differ from P35 males ($p = 0.701$; see Fig. 2-3C). There was no correlation between corticosterone and progesterone at any age after recovery,

whereas hormone concentrations were positively correlated immediately post-restraint (Experiment 1a).

GR translocation

Thirty minutes post-restraint, there were age-related differences in the expression of GR in the cytosolic ($F_{2,19}$ = 6.06, p = 0.009) and nuclear ($F_{2,19}$ = 4.48, p = 0.025) fractions in the hippocampus. In the cytosolic fraction, P35 males did not differ from P45 males (p = 1.0) and had greater expression than did P75 males (p = 0.009). There was also a trend for P45 males to have greater expression of GR in the cytosol than P75 males (p = 0.078), but this did not reach statistical significance. Similar to our findings immediately post-restraint (Experiment 1a), there was greater expression of GR in the nuclear fraction in P35 males compared to P75 males (p = 0.028) and neither P35s nor P75s differed from P45 males (ps = 0.167 and 1.0, respectively; see Fig. 2-3D).

Experiment 2

RT-qPCR

For the expression of *NR3C1* (GR) mRNA in the hippocampus, there was no effect of age (p = 0.354), but there was a significant effect of time-point ($F_{1,42}$ = 15.00, p < 0.001), which did not depend on age (p = 0.728); compared with baseline, *NR3C1* expression was lower post-restraint (see Fig. 2-4A). For *NR3C2* (MR) expression, there was no effect of age (p = 0.267), of time-point (p = 0.477), or interaction of the two (p = 0.460; see Fig. 2- 4B). We also measured the expression of receptor chaperones in the hippocampus and found that *FKBP5* did not differ between the different age groups (p = 0.211), although there was a significant effect of time-point ($F_{1,41}$ = 7.33, p = 0.010), which was independent of age (p = 0.931); compared with

baseline, *FKBP5* expression was greater post-restraint (see Fig. 2-4C; note that one post-restraint P35 sample was dropped from the analysis because it amplified inappropriately). In contrast, we did find a significant effect of age on the expression of *FKBP4* ($F_{2,42} = 4.39$, $p = 0.019$) and relative to baseline, expression was increased post-restraint, although the difference failed to meet the criteria for statistical significance ($F_{1,42} = 3.53$, $p = 0.067$). There was no interaction of age and time-point on *FKBP4* expression (p = 0.398). Post hoc comparisons indicated that, for *FKBP4*, P35 males had greater expression than did P75 males ($p = 0.019$) and P45 males did not differ from either age group (ps = 0.132 and 1.0, respectively; see Fig. 2-4D). For the expression of *Bag1*, there was no effect of age ($p = 0.247$), nor of time-point ($p = 0.577$), nor was there an interaction of the two factors ($p = 0.839$; see Fig. 2-4E).

Discussion

We investigated age-related differences in HPA function by comparing pre-pubertal and post- pubertal adolescents with adult males on neuroendocrine function and on GR translocation in response to an acute stressor. We also assessed mRNA expression for corticosteroid receptors (GR and MR) and receptor chaperones (Fkbp51, Fkbp52 and Bag1), which are known to modulate GR activity. The data indicated that, during the adolescent period, there are marked developmental shifts in the stressor-induced release of hormones (i.e., progesterone, corticosterone, and testosterone) and in GR translocation, which were likely unrelated to chaperones, as we found little evidence for developmental shifts in their expression.

Based on previous research, we predicted that pre-pubertal adolescents would have an exaggerated and prolonged corticosterone response to an acute stressor compared

with that of adults (Goldman et al., 1973; Vazquez and Akil, 1993; Romeo et al., 2004a, 2006b, 2014; Lui et al., 2012; Hall and Romeo, 2014). Consistent with our prediction and the literature, we found that P35 males had greater post-stress concentrations of corticosterone compared with P75 males. In post-pubertal adolescent rats (P45), for which less is known about HPA function, concentrations after restraint stress were intermediate to those of pre-pubertal adolescent and adult rats. We did not find evidence of a protracted corticosterone response in adolescent rats, however; concentrations of corticosterone had returned to basal titers (~5–25 ng/mL) after 30 min of recovery, which suggests efficient HPA feedback in all three age groups.

Concentrations of progesterone after restraint stress were markedly higher in both groups of adolescent rats compared with adult rats, which is consistent with what was found in pre-pubertal adolescents compared with adults (Romeo et al., 2004b, 2005, 2006b). Age-related differences in the release of progesterone in response to a stressor are not related to gonadal status based on several lines of evidence. Firstly, in the present study P45 males had greater post-stress concentrations of progesterone despite having adult-like concentrations of testosterone at baseline. Secondly, both pre-pubertal males and females had greater stressor-induced release of progesterone and corticosterone compared with that of adults when rats of both ages were gonadectomized (Romeo et al., 2004a,b, 2005), thus ruling out circulating gonadal hormones as an explanation for age-related differences. Instead, adolescent shifts in progesterone and corticosterone responses to stress may be related to ongoing adrenocortical development, as adrenalectomy blocks the stress-induced increase in their release (Hueston and Deak, 2014). Previous studies have shown that, compared with adults, pre-pubertal adolescents

have larger adrenals after normalizing to body weight and have elevated mRNA expression of the melanocortin 2 receptor, which may confer greater sensitivity to ACTH (Foilb et al., 2011; Lui et al., 2012; Romeo et al., 2014). Also, our findings that progesterone and corticosterone were positively correlated in all age groups after stress is consistent with what has been reported in adult male rats and is explained by the fact that ACTH acts as a secretagogue of both hormones (Hueston and Deak, 2014). Nevertheless, developmental changes in adrenal function cannot fully account for the exaggerated stress responses of pre-pubertal adolescents given that ACTH release (Romeo et al., 2004a, 2006a,b; Hall and Romeo, 2014) and PVN activation are also more pronounced in response to a stressor in adolescents compared with adults (Romeo et al., 2006a; Hodges et al., 2014).

Little is known about the function of progesterone release in response to a stressor; there is some evidence that progesterone dampens HPA function and reduces anxiety- and depressive-like behaviour (Viau and Meaney, 1991; Frye, 2011; Schiller et al., 2014). The implications of age-related differences in stressor-induced progesterone release on physiology and behaviour are unknown, but warrant investigation. Progesterone can competitively bind to GR and MR and act as a partial agonist (Rousseau et al., 1972; Rupprecht et al., 1993), and GR and progesterone receptors have both common and unique binding sites on DNA (Hubler and Scammell, 2004).

In addition to finding age-related differences in baseline testosterone (pre-pubertal adolescent males had lower concentrations than did post-pubertal adolescents and adults), the effect of stress on the release of testosterone differed by age; stress decreased testosterone concentrations in adolescents and increased concentrations in adults. These

findings are consistent with reports that acute stress increased testosterone concentrations in adults (Armario and Castellanos, 1984; Romeo et al., 2004a; Foilb et al., 2011; Bourke et al., 2013; Hodges et al., 2014), and decreased concentrations in pre-pubertal adolescents (Romeo et al., 2004a; Foilb et al., 2011), and our research extends the results to post-pubertal adolescents. Further, acute stress has been shown to increase plasma concentrations of testosterone in adult baboons (Sapolsky, 1986) and humans (Girdler et al., 1997), and in juvenile, pubertal, and adult birds (Heiblum et al., 2000); thus the dampening effect of stress on testosterone in adolescents may be specific to rats. It is unclear why adolescent rats have lower concentrations of testosterone after stress, but others have speculated that there may be an initial release of testosterone that triggers a subsequent reduction in adolescent rats because of heightened feedback during this developmental window (Klein and Romeo, 2013). Although evidence suggests that stressors begin to increase testosterone release at approximately 50 days of age (Foilb et al., 2011), the molecular mechanisms that underlie shifts in HPA and HPG interactions (i.e., stressor effects on testosterone, and testosterone effects on stress responses) from that found in adolescence to that found in adulthood are unknown.

Previous reports that adolescent rats have immature HPA negative feedback (Goldman et al., 1973; Vazquez and Akil, 1993) led us to investigate GR activity in the hippocampus, although we found that rats of all ages had recovered by 30 min after the stressor. Nevertheless, others have speculated that GR translocation is different in pre-pubertal adolescents compared with adult rats (Dziedzic et al., 2014). We selected the hippocampus because it richly expresses corticosteroid receptors and is involved in regulating HPA function (Herman et al., 2012), and the involvement of the hippocampus

in HPA feedback is mediated in part by classical GR actions (Feldman and Weidenfeld, 1999; Furay et al., 2008). Furthermore, there is some evidence of age-related differences in translocation; when dexamethasone was injected into the hippocampus of young adult rats, GR translocation increased and basal plasma corticosterone decreased, but in aged rats, dexamethasone administration did not induce translocation, nor did it reduce corticosterone (Mizoguchi et al., 2009).

Although others have reported no age-related difference in GR expression (Dziedzic et al., 2014; Romeo et al., 2008), they had not investigated cytosolic and nuclear GR separately. We did not find age-related differences in baseline expression of GR in the hippocampus within the cellular compartments examined (i.e., cytosolic and nuclear). We did, however, find evidence of GR translocation in that restraint stress decreased receptor expression in the cytosol and increased expression in the nucleus, and the magnitude of change depended on age. Contrary to expectations, pre-pubertal adolescent males had more GR translocation compared with that of adults as indicated by greater expression of nuclear GR immediately after restraint stress, which corresponded with, and likely reflected, the observed age-related difference in the release of corticosterone. Consistent with this interpretation, as was found for plasma corticosterone concentrations, GR translocation in post-pubertal adolescents was intermediate to that of pre-pubertal adolescents and adults. Further, age-related differences in the release of progesterone in response to a stressor may have contributed to the age-related difference observed in GR translocation, as progesterone has been shown to bind GR and trigger translocation (Rupprecht et al., 1993; Peeters et al., 2008). Unlike in the nuclear fraction of hippocampal tissue, we did not find an age-related difference in GR expression in the

cytosol. Nevertheless, the change from baseline to post-restraint appeared to be more pronounced in P35 males than in P45 and P75 males (Fig. 2-2B and 2-2D), consistent with what was seen in the nucleus (Fig. 2-2C and 2-2E), and the lack of a significant interaction is likely related to small group sizes and reduced sensitivity to detect an effect of stress in the cytosol relative to that seen in the nucleus. A question that remains is why P35 males had elevated GR expression in both the cytosolic and nuclear fractions 30 min after cessation of the stressor compared with that of adults. One possibility is that the greater expression in the nucleus reflects persistent age-related difference in translocation, whereas differences in total (nuclear and cytosolic) GR may be related to age-related differences in protein degradation after a stressor. Future studies could assess developmental shifts in degradation by measuring GR translocation after a stressor in the presence of a proteasome inhibitor.

Because we had predicted age-related differences in GR translocation, we investigated mRNA expression of chaperones that modulate GR signaling (i.e., *FKBP5, FKBP4, BAG1*) as well as mRNA expression of corticosteroid receptors (i.e., *NR3C1* and *NR3C2*). Only the expression of FKBP4 (codes for Fkbpp52, a pro-translocation chaperone) differed as a function of age and in the direction opposite of what we had predicted; we found that pre-pubertal adolescents had greater expression than adults. Nevertheless, this may provide correlational evidence of why adolescents had more, not less, translocation relative to that of adults, although future studies are needed to identify Fkbp52 as a mechanism. Independent of age, stress altered the expression of several genes of interest in the hippocampus including *NR3C1* (GR), which was down-regulated in all age groups 90 min after termination of restraint, consistent with findings in the

literature that stress decreases its expression in the hippocampus of adults (Herman, 1993; Romeo et al., 2008; Noguchi et al., 2010) and of pre-pubertal adolescents (Romeo et al., 2008). Further, we found that stress up-regulated *FKBP5* mRNA, consistent with the previous reports in rats (Bourke et al., 2013), mice (Scharf et al., 2011), or via application of glucocorticoids in cell culture (Vermeer et al., 2003). The stress-induced increase in *FKBP5* may represent an 'ultra-short negative feedback loop' given that it reduces GR's affinity for glucocorticoids and ability to translocate into the nucleus (Binder, 2009).

In sum, pre-pubertal adolescents had greater HPA responses and GR translocation than did adults despite equal effects of stress at both ages on *NR3C1* and *FKBP5* mRNA, which suggests that there are age-related differences in their regulation by stress. The lack of an age-related difference in mRNA expression post-restraint is surprising based on the differences in corticosterone and GR translocation, because previous studies reported that changes in mRNA expression are related to the extent of corticosterone release; the expression of *NR3C1* in the hippocampus of adrenalectomized rats (Hugin-Flores et al., 2004) and *FKBP5* in cell culture (Vermeer et al., 2003) is dose-dependently regulated after treatment with a GR agonist. There are many factors that might contribute to age-related differences in GR activity inside the nucleus. For example, there may be age-related differences in co-factors (e.g., GR interacting protein [GRIP1] or steroid receptor co-activator 1 [SRC-1]) that modulate GR activity inside the nucleus; expression of *SRC-1* was shown to change across the adolescent period in the female rat brain and is modulated by gonadal hormones (Mitev et al., 2003). Alternatively, greater release of progesterone in adolescent rats may have resulted in differential transcriptional properties

of GR, as progesterone induces GR translocation without having the same transcriptional properties as that of GR bound to glucocorticoids (Rupprecht et al., 1993).

One limitation of the experiments is that rats were shipped from a commercial supplier, and the effects of shipping stress in rats may be age-specific. We did not find differences in baseline corticosterone across the age groups, which may indicate that shipping stress did not produce any age-specific lasting effect on the HPA axis. In addition, others have reported that age-related differences in HPA responses to stressors did not depend on whether the rats were shipped or obtained in-house (Lui et al., 2012). Nevertheless, we cannot rule out an effect of shipping stress from our results.

In conclusion, neuroendocrine responses to acute stressors change significantly during the adolescent period. Despite increased gonadal function, the post-pubertal adolescent's responding resembles more that of pre-pubertal adolescents than adults. The greater stressor-induced GR translocation in pre-pubertal adolescents may contribute to the enhanced sensitivity of adolescent rats to the effects of chronic stress exposures compared with adults (McCormick and Green, 2013).

Tables

Table 2-1. Sequences of primers used in RT-qPCR reactions.

Gene	Forward primer	Reverse Primer
NR3C1 (GR)	GTGCTACCCCGACAGAGAAG	CCGCCAAAGGAGAAAGCAAG
NR3C2 (MR)	CAGCTCACCTCCATTACGCA	CTTCACGACCTGGCTCATCT
FKBP5	TTCCCTCGAACGCAACTCTC	GTCGTGGTCTTCTCCTTCGC
FKBP4	GAAGTCGGGGAAGGGGAAAG	GCATGTGGTGGGATCTGGAA
BAG1	TAGCGGAAGAGGTGACCCAG	CTCCTGTGGCCTCTTCAACA
Cyclophilin A	CCGCTGTCTCTTTTCGCC	ATCCTTTCTCCCCAGTGCTC
GAPDH	GGAAGCTGGTCATCAACGGG	CGGAGATGATGACCCTTTTGG

Figures

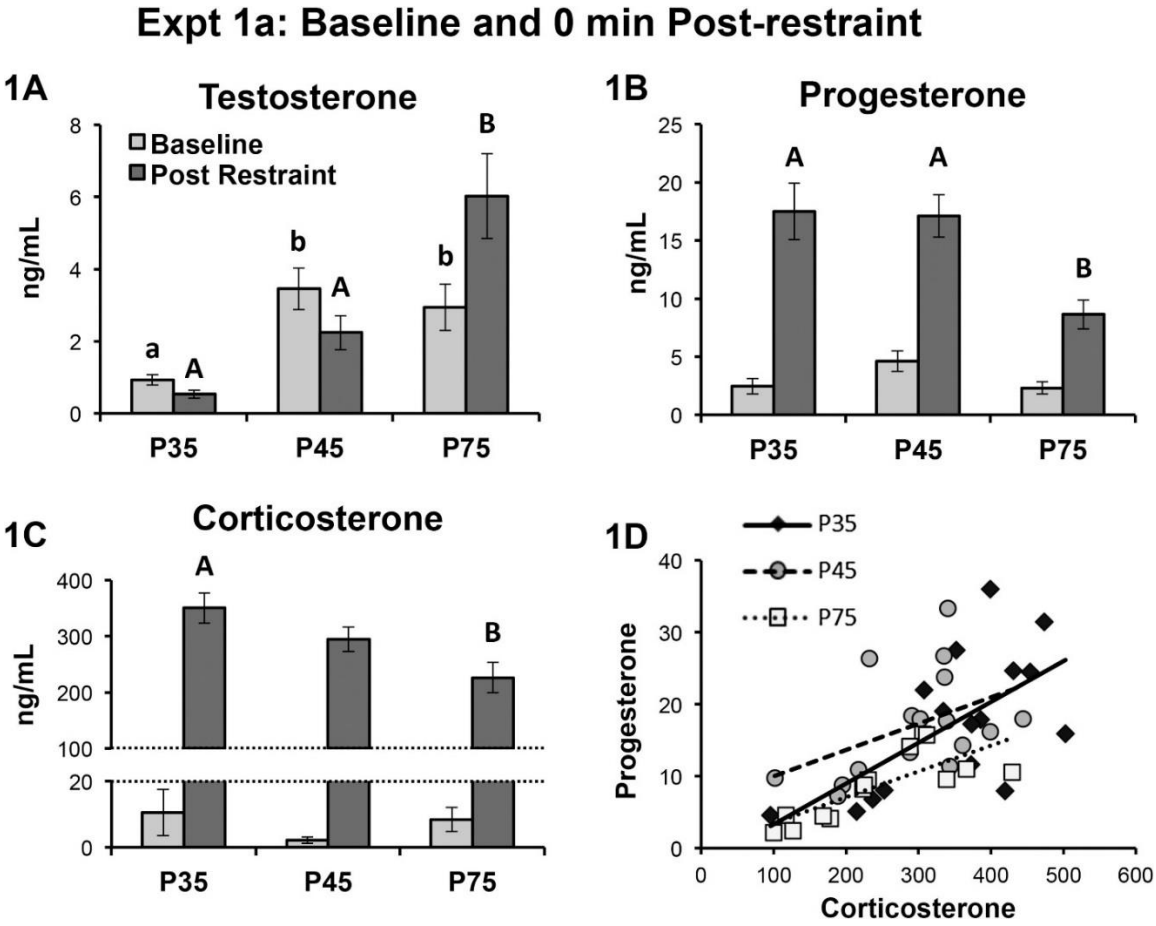

Fig. 2-1. Mean (±S.E.M.) plasma hormone concentrations of (A) testosterone, (B) progesterone, and (C) corticosterone immediately before (baseline) and after (post-restraint) 30 min of restraint stress in P35, P45, and P75 male rats. (D) Depicts a positive correlation between plasma corticosterone and progesterone post-restraint in male rats at P35 (r = 0.63), P45 (r = 0.44), and P75 (r = 0.78). Different lowercase letters denote significant differences between ages in baseline concentrations ($p < 0.05$). Different uppercase letters denote significant differences between ages in post-restraint concentrations ($p < 0.05$).

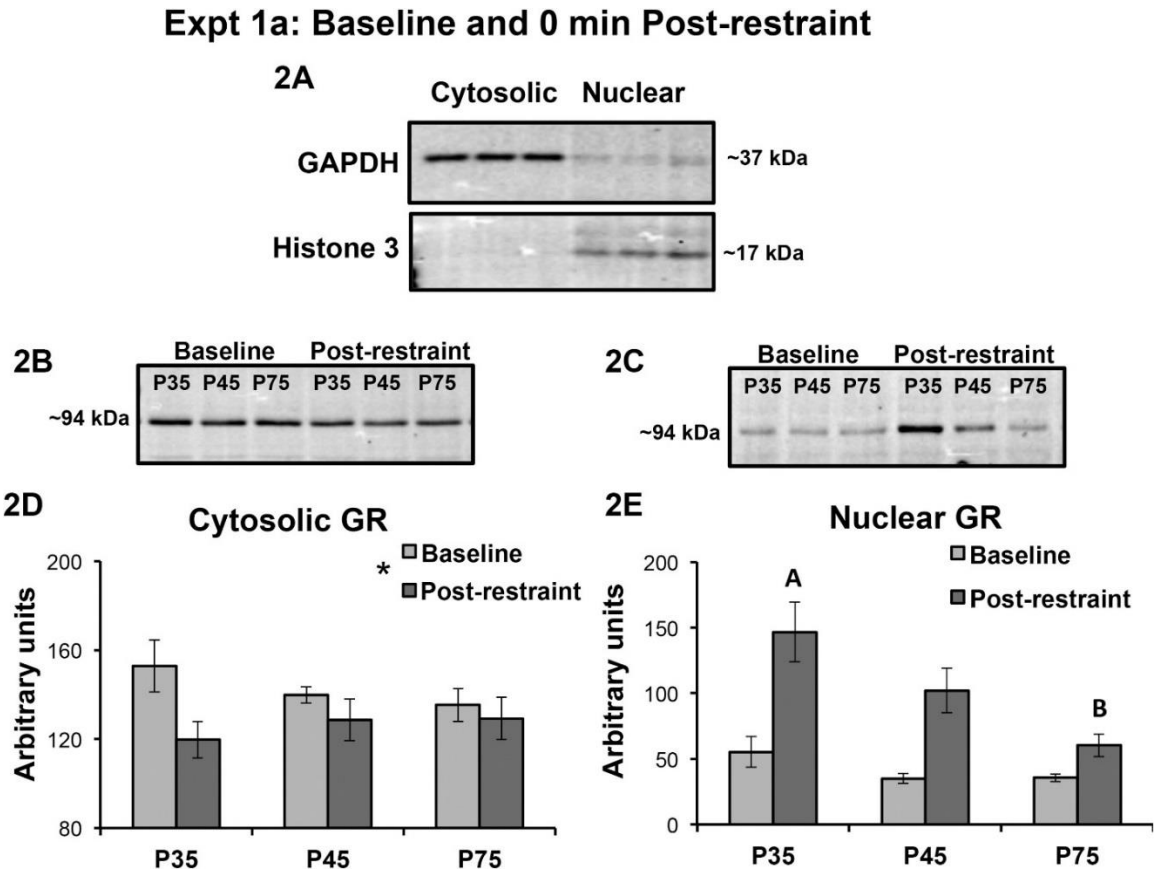

Fig. 2-2. (A) Representative Western blot demonstrating successful isolation of cytosolic and nuclear fractions in hippocampal tissue using anti-GAPDH and anti-H3 antibodies, respectively. Representative Western blot of GR protein expression in the (B) cytosolic and (C) nuclear fractions. Mean (±S.E.M.) expression of GR protein in the (D) cytosolic and (E) nuclear fractions immediately before (baseline) and after (post-restraint) 30 min of restraint stress in P35, P45, and P75 male rats. All data are expressed in arbitrary units. Different uppercase letters denote significant age-related differences post-restraint and an asterisk indicates a significant main effect of time-point ($p < 0.05$).

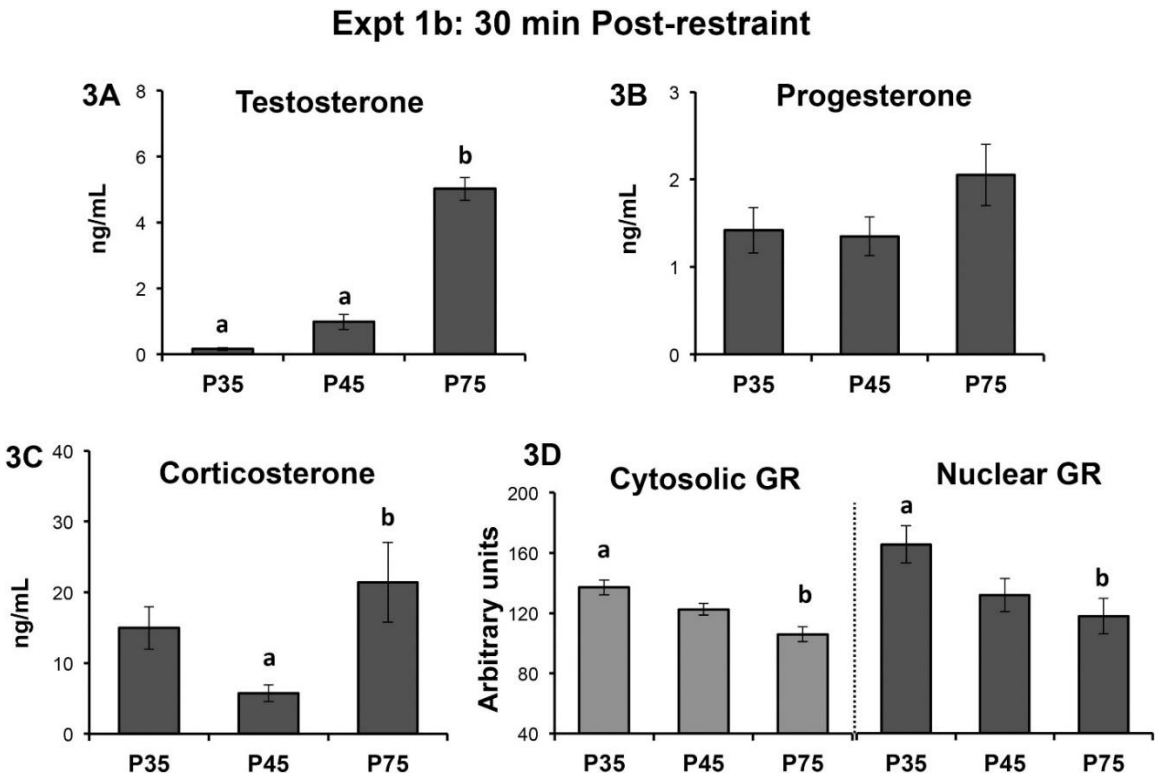

Fig. 2-3. Mean (±S.E.M.) plasma hormone concentrations of (A) testosterone, (B) progesterone, and (C) corticosterone 30 min after the cessation of restraint stress in P35, P45, and P75 male rats. (D) Mean (±S.E.M.) expression of cytosolic and nuclear GR protein 30 min after the cessation of stress in P35, P45, and P75 male rats. Different lower case letters denote significant age-related differences ($p < 0.05$).

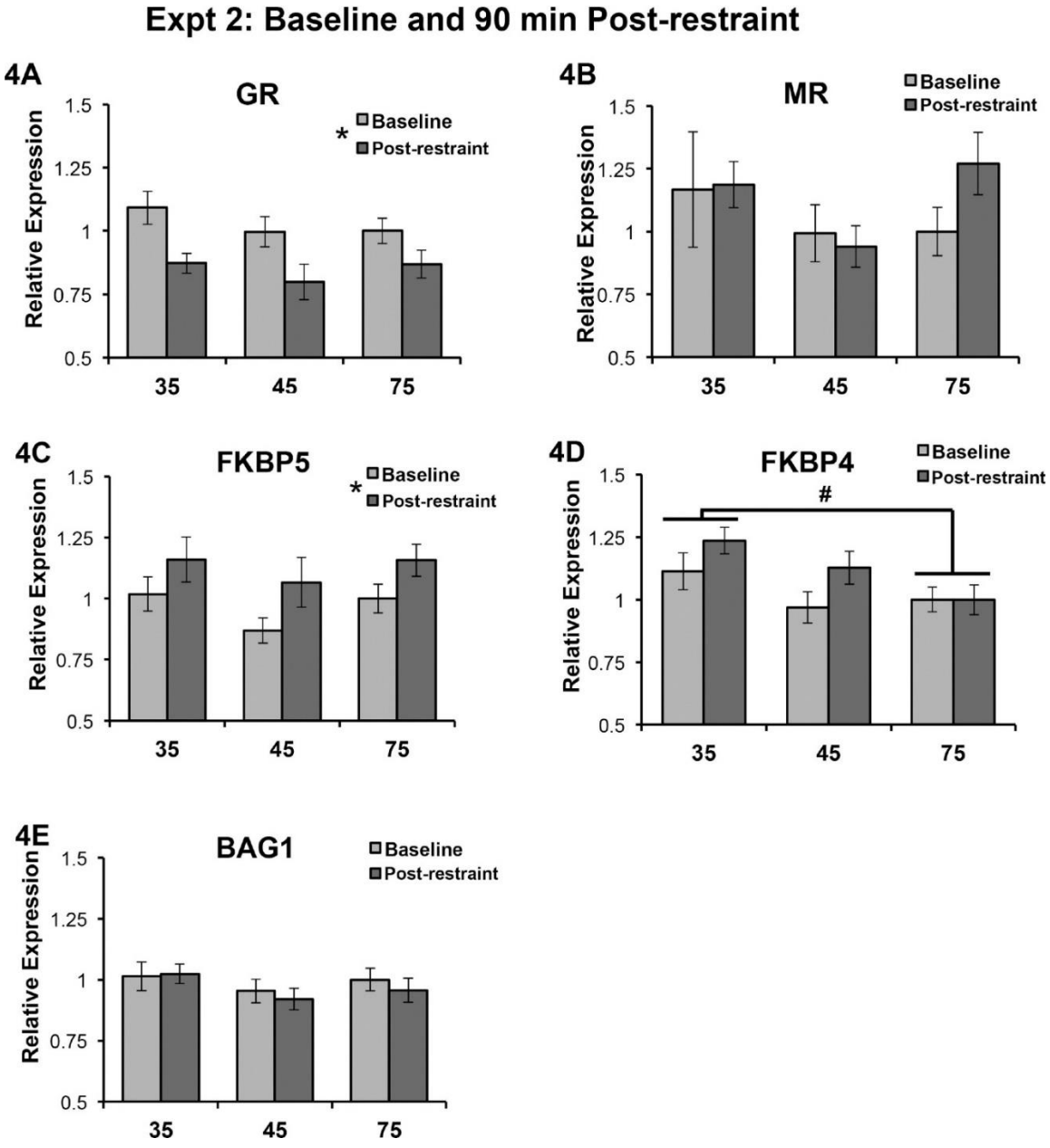

Fig. 2-4. Mean (±S.E.M.) mRNA expression of (A) *GR*, (B) *MR*, (C) *FKBP5*, (D) *FKBP4*, and (E) *BAG1* immediately before (baseline) and 90 min after (post-restraint) 30 min of restraint stress in P35, P45, and P75 male rats. Data are expressed as fold change relative to baseline P75 rats. An asterisk denotes a significant main effect of time-point and a number sign denotes significant difference between ages ($p < 0.05$).

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Rationale for Chapter 3

In chapter 2, I provided evidence of developmental shifts in HPA function; pre-pubertal and post-pubertal adolescent males had greater release of corticosterone and progesterone in response to restraint stress than did adult males. I also showed that these differences were not likely related to differential GR-mediated negative feedback in the hippocampus or expression of co-chaperones known to modulate GR activity. An alternative possibility is that age-related differences in HPA responses are related to changes in circulating gonadal hormones. In adult males, androgens reduce HPA responses to stress and facilitate a return to baseline concentrations of corticosterone (Green & McCormick, 2016). Therefore, pre-pubertal males may respond more strongly, because adults have markedly higher concentrations of circulating testosterone. One previous study addressed this question and found that the age-related differences persisted when both age groups were OCXed and given a testosterone implant (Romeo et al., 2004). Therefore, in the next chapter, I sought to replicate this study and further investigate gonadal status as a source of age-related differences in HPA reactivity.

If testosterone cannot make the pre-pubertal HPA response adult-like, then this suggests that males at this age are unresponsive to the dampening effects of testosterone, and begs the question of when does the HPA axis become sensitive to gonadal hormones. In this chapter, I compare the effects of a testosterone implant (versus those given a blank implant) at different ages to examine developmental shifts in HPA regulation by gonadal hormones. In addition to measuring corticosterone concentrations (the main output of the HPA axis), I examined Fos and AVP expression in the PVN, where the hormonal stress response is initiated. Fos is a marker of neuronal activation and AVP is a co-

sectretagogue of ACTH, which triggers the release of corticosterone from the adrenals. In adults, both Fos and AVP are dampened by testosterone (Goel et al., 2014).

In this chapter, I also conduct a second experiment to replicate and extend the findings from experiment 1; we found that the effects of testosterone were different at each age examined. Actions of testosterone are mainly attributed to activation of androgen receptors (AR); however, testosterone can also be converted to estradiol or to DHT, which can be further converted to 3β-diol. In adult males, estradiol increases HPA function by binding to estrogen receptor α (ERα), whereas DHT and 3β-diol reduce HPA function by binding to AR or estrogen receptor β (ERβ), respectively (Handa & Weiser, 2014). Therefore, age-specific effects of testosterone on HPA function may be related to developmental changes in enzymes that regulate testosterone conversion. To tease apart the contributions of receptor targets on modulating HPA function in the three age groups, I examine the effects of AR and ER antagonism (versus vehicle) in OCX males that received testosterone or DHT on concentrations of corticosterone after exposure to an acute stressor.

Chapter 3: The effects of gonadal hormones on HPA function change throughout adolescence and do not account for developmental shifts in stress reactivity.

Introduction

Developmental changes in hypothalamic-pituitary-adrenal (HPA) function during adolescence have been well documented in rodents (Romeo, 2013; Green & McCormick, 2016). Pre-pubertal adolescents respond to an acute stressor with a more pronounced release of adrenocorticotropic hormone (ACTH) and corticosterone than do adults. Moreover, concentrations remain elevated for longer in pre-pubertal males, despite similar rates of hormone metabolism (Schapiro, Percin, & Kotichas, 1971; Vazquez, 1998), which suggests that negative feedback is maturing during adolescence (Green & McCormick, 2016; Romeo, 2013). The underlying mechanisms that mediate developmental shifts in HPA function, however, remain somewhat unclear and may be related to actions at each level of the HPA axis.

Relative to total body weight, the adrenal glands of pre-pubertal adolescents are larger than that of adults and may be more sensitive to ACTH (Romeo et al., 2014). Therefore, age-related differences may be in part related to developmental shifts in adrenal structure and function. Nevertheless, pre-pubertal adolescents also release more ACTH in response to a stressor and have greater activation of the paraventricular nucleus (PVN) of the hypothalamus (Hodges & McCormick, 2015; Lui et al., 2012; Romeo et al., 2006; Viau, Bingham, Davis, Lee, & Wong, 2005), which indicates developmental changes in the brain and possibly pituitary. Another possibility is that differences in HPA function are explained by age-related changes in gonadal status. In adult males, testosterone and the more potent androgen, dihydrotestosterone (DHT), have been shown to suppress the corticosterone response to stress and facilitate a return to baseline concentrations (Goel, Workman, Lee, Innala, & Viau, 2014; Handa & Weiser, 2014).

The suppressing effects of testosterone on circulating concentrations of corticosterone are likely unrelated to steroid clearance (Kitay, 1963a, 1963b) and, like age-related differences, can be observed at each level of the HPA axis (Green & McCormick, 2016; Romeo, 2013). There is more evidence for actions of androgens in the brain and pituitary, in part related to actions on androgen receptors (AR), which is supported by evidence that flutamide (an AR antagonist) increases stress responses in intact or androgen treated males (Gomez, Manalo, & Dallman, 2004; Lund, Hinds, & Handa, 2006). The neuroendocrine cells of the PVN are devoid of AR, however, which suggests that androgenic effects occur in upstream brain regions. The medial preoptic area (mPOA), which richly expresses AR and has connections to the PVN, is an important site for androgenic effects on HPA function. Direct administration of androgens to the mPOA reduced post-stress concentrations of corticosterone (McCormick, Linkroum, Sallinen, & Miller, 2002) and expression of Fos and AVP heteronuclear (hn)RNA in the PVN (Williamson, Bingham, Gray, Innala, & Viau, 2010), and lesions to this region prevented the dampening effects of testosterone (Viau & Meaney, 1996).

In addition to actions at AR, androgen metabolites can directly affect PVN function and possibly upstream brain regions by acting on alternative receptor targets. It is well established that testosterone can be converted irreversibly to DHT via 5α-reductase or to estradiol via aromatase (Handa & Weiser, 2014). Because DHT cannot be aromatized into estradiol, and because estradiol increases rather than suppresses HPA function via estrogen receptor α (ERα) (Lund et al., 2006; Lund, Munson, Haldy, & Handa, 2004), this pathway is not a likely candidate for the suppressing effects of

androgens. There is evidence, however, that DHT can be irreversibly converted to 3β-diol, which can reduce HPA activation by binding to estrogen receptor β (ERβ) (Handa, Pak, Kudwa, Lund, & Hinds, 2008; Lund et al., 2006), a receptor that is expressed within a small population of neuroendocrine cells in the PVN, among other stress-related brain regions (Handa & Weiser, 2014).

Given that pre-pubertal adolescents have low levels of circulating androgens compared with that of adults, it seems likely that age-related differences in gonadal status account for the pronounced HPA responses seen before puberty. Contrary to this hypothesis, one study found that pre-pubertal adolescents continued to exert a more pronounced stress response than adults after both groups were OCXed (to eliminate testicular androgens) and given a comparable dose of testosterone (Romeo, Lee, Chhua, McPherson, & McEwen, 2004). These findings indicate that developmental shifts in HPA function are likely independent of differences in gonadal status. Therefore, in the present study, we attempted to replicate the finding that gonadal status is not the basis for age-related differences in HPA function. Moreover, we investigated whether gonadal hormones can regulate HPA function before puberty, as the study by Romeo and colleagues (2004) examined age-related differences in the presence of testosterone, but did not examine testosterone's effects at each age (i.e., no comparison with non-replaced males), which would provide a better indication of testosterone's ability to dampen HPA function at each age. We included post-pubertal adolescent males (P45) that are still maturing and for which far less is known regarding HPA function, to better understand the transition into adulthood. To quantify HPA reactivity and recovery we measured plasma corticosterone concentrations as well as Fos (a marker of neuronal activation) and

AVP (a co-secretagogue of ACTH) expression in the PVN, all of which are dampened by androgen treatment in adulthood (Goel et al., 2014; Handa & Weiser, 2014).

Experiment 1: Effects of GDX and testosterone replacement on HPA function before during and after puberty in male rats.

Methods

Animals

Male Long-Evans rats were obtained at postnatal day (P) 24 (n = 48), P34 (n = 32), and P64 (n = 32) (± 1 day) from Charles River (St. Constant, Quebec) in two batches, which were balanced for age. Animals were housed in same-aged pairs and acclimated to the colony for at least 4 days. Food and water was provided ad libitum and animals were kept on a 12 h light-dark cycle (lights on at 09:00). All procedures were approved by the Brock University Animal Care and Use Committee and were in keeping with the National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) as well as the Canadian Council on Animal Care guidelines.

Orchiectomy and testosterone treatment

After the acclimation period, all males underwent an orchiectomy (OCX) surgery under isoflurane. Surgery occurred across 4 days, with 8 males from each age group receiving an OCX on each day (n = 32 per age). On days 3 and 4 of surgery, an additional 8 males from the youngest age group underwent OCX (n = 48); we attempted to reduce the number of animals needed for the study by using repeated blood sampling (described below), but pilot experiments indicated that males at the younger age were too small to extract repeated samples from and thus, we included more males. After OCX, but while

still under isoflurane, half the males in each age group received a subcutaneous silastic tube implant that contained ~10 mg of testosterone. The other halves received a blank implant.

Stress and sample collection

Four days after surgery, males from each age group were euthanized via rapid decapitation for the collection of brain tissue and trunk blood. Samples from those that underwent surgery on day 1 or 2, were collected directly from the home cage (baseline time-point) approximately 1 to 1.5 hours into the lights on phase of the light-dark cycle (*n* = 8 per age and hormone treatment) (see Fig. 3-1). The remaining males (those that had surgery on day 3 or 4), underwent 30 min of restraint stress in a Plexiglas® restrainer, which began 30 min into the lights-on phase. Because of the age-related differences in the size of the males, we utilized 3 different sizes of restrainer that permitted minimal mobility. Immediately after restraint stress (0 min post-stress time-point), the older age groups had blood samples taken from the tail vein within a minute, then were returned to their home-cage. Based on pilot data, we could not extract a sufficient amount blood from the tail vein of the younger group to perform hormone assays and thus, half of the animals from this age were euthanized via rapid decapitation for the collection of trunk blood. The remaining half of the younger cohort underwent the same tail snip and handling as the older groups, but was returned to the home-cage for the future collection of brains and trunk blood, which occurred 45 min after termination of the stress procedure (45 min post-stress time-point). This time-point was selected based on previous studies showing age-related differences in corticosterone and in Fos expression in the PVN between 0 and 45 min after 30 min of restraint stress (Lui et al., 2012; Romeo

et al., 2006). Additionally, these studies found that pre-pubertal adolescents reached peak expression as early as immediately after stress, whereas adults may take longer (Lui et al., 2012; Romeo et al., 2006). Therefore, we waited until 45 min after the end of restraint to be more confident that any age effect would not be related to differences in the speed at which the groups reached maximal expression. All blood samples were collected into ice-chilled tubes containing EDTA, spun for 10 min at 1,730 RCF and 4˚C for the collection of plasma, which was kept at -20˚C until hormones assays. Brains were rapidly extracted and sliced on ice along the coronal plane directly rostral and caudal to the hypothalamus and the sections were fixed via submersion in 4% paraformaldehyde containing 10%, then 20%, then 30% sucrose (~24 hours per concentration of sucrose). After fixation, brains were frozen for 1 hour at -20˚C, then sliced into 40 µm coronal sections, with every fifth section pooled. Sections were stored in cryoprotectant at -20˚C until immunohistochemistry experiments for the detection of Fos and AVP immunoreactive (ir) cells in the medial parvocellular PVN.

Hormones assay

Steroids were extracted from plasma (25 µL for tail vein samples, 100 µL for trunk samples) using diethyl ether and the samples were reconstituted in buffer provided in the enzyme-linked immunosorbent assay kits for the detection of testosterone, corticosterone and progesterone (Neogen, Lansing, MI). Assay procedures were conducted as per kit instructions and sensitivity was 0.006, 0.05, and 0.4 ng/mL for testosterone, corticosterone, and progesterone, respectively. Concentrations of corticosterone were measured from samples collected at each of the 3 time-points to access stress reactivity and recovery. Testosterone concentrations were assayed in

baseline samples only and from those OCX males that received testosterone-replacement; pilot data indicated that concentrations were below the limit of detection in blank implant treated OCX males. For progesterone, baseline concentrations were assayed in males given a testosterone or blank implant.

Immunohistochemistry

Briefly, free-floating sections were washed thoroughly in 0.1 M PBS, then in PBS-X (0.1 M PBS with 3% Triton X-100), and incubated at room temperature in a 0.3% H_2O_2 in 0.1 M PBS-X solution for 30 mins. Sections were then washed in PBS-X, blocked at room temperature in 10% goat serum (Sigma) solution for 1 h, and incubated at 4 °C overnight in rabbit anti-AVP (1:30,000; Santa Cruz) or rabbit anti-Fos (1:20,000; Santa Cruz) in PBS-X. The next day, sections were washed in PBS-X and then incubated for 2 h at room temperature in a biotinylated goat anti-rabbit (1:4000; Vector Laboratories). After another series of washes in PBS-X, sections were incubated in an avidin–biotin horseradish peroxidase complex (Vector Laboratories) for 1.5 h at room temperature. Horseradish peroxidase was visualized with 3,3′-diaminobenzidine (DAB) in a 3 M sodium acetate buffer containing 0.05% H_2O_2 (Vector Laboratories). After a final series of washes in PBS-X, sections were mounted on Superfrost Plus slides (Fisher Scientific) and allowed to dry. Mounted sections were dehydrated using increasing concentrations of ethanol (70%, 95%, 100%, 3 mins each), then were placed in xylene (~3 mins), and coverslipped using Permount mounting medium (Fisher Scientific).

Microscopy and cell counting

Immunostained sections were imaged using a Nikon Eclipse 80i microscope equipped with a digital camera (Nikon DXM1200F) and Nikon ACT-1 software. Fos-ir

and AVP-ir cell counts were conducted at 400× magnification in a 250 μm^2 area within each hemisphere of the medial parvocellular division of the paraventricular nucleus (PVN), where input from various stress-responsive brain regions is integrated to mount a hormonal stress response. Counts were made blind to experimental condition and the mean number of ir-cells were scored, which was taken by averaging counts from both hemispheres and across multiple sections (if present).

Statistical analyses

Analyses involved 1-way (for testosterone; age), 2 factor (for progesterone; age, treatment) and 3 factor (for corticosterone, Fos, and AVP; age, treatment, time-point) ANOVAs. Alpha was set at $p < 0.05$ and post hoc tests were Bonferroni-corrected.

Results

Hormones

Testosterone concentrations were ~2 ng/mL in OCX males given testosterone implants, and did not differ by age ($F_{2,21} = 0.2$, $p = 0.825$; see Fig. 3-2A). This concentration corresponds to approximately low-to-mid physiological concentrations of testosterone (Viau & Meaney, 1996) and is similar to those in sham-operated, adult males (Seale, Wood, Atkinson, Harbuz, & Lightman, 2004).

Baseline concentrations of progesterone differed by age ($F_{2,42} = 5.5$, $p = 0.007$; see Fig. 3-2B); P35 males had greater concentrations than did P45 males ($p = 0.006$), and P75 did not differ from either age group (ps = 0.476 and 0.203, respectively). The effect of age did not depend on treatment condition ($F_{2,42} = 0.1$, $p = 0.865$). Testosterone replacement increased baseline concentrations of progesterone, but the effect was not statistically significant ($F_{1,42} = 3.2$, $p = 0.081$).

There was a significant interaction of age, treatment, and time-point on concentrations of corticosterone ($F_{4,119} = 5.6$, $p < 0.001$). Follow-up analyses were split by time-point, which indicated no effect or interaction of age and treatment at baseline or at 45 min post-stress (all *p*s > 0.1) (see Fig. 3-2C, left and right panels). At the recovery time-point, concentrations approached baseline (<5 μg/dL) in all groups. Based on previous research (Romeo et al., 2004), we predicted that the effect of testosterone on corticosterone concentrations immediately after stress would depend on age. Consistent with our predictions we found a significant interaction of the 2 factors ($F_{2,38} = 5.3$, $p = 0.009$). Therefore, we conducted follow-up analyses split by treatment and by age to examine developmental changes in HPA function and its modulation by testosterone.

Testosterone replacement had no effect on post-stress concentrations of corticosterone at P35 ($p = 0.690$) or at P75 ($p = 0.150$), but increased concentrations at P45 compared with those that received blank implants ($t_{13} = 3.0$, $p = 0.010$) (see Fig. 3-2C, middle panel). The lack of a difference at P75 was unexpected considering that testosterone typically dampens stress reactivity in adulthood (Goel et al., 2014; Handa & Weiser, 2014); our data, however, are in the same direction and involved small groups (6-7 per treatment at P75). When we split by treatment, we found evidence of developmental shifts in HPA function; among OCX males given a blank implant, P35 males had higher concentrations than did P45 ($p = 0.003$) and P75 males ($p = 0.033$), but there was no difference between P45 and P75 males ($p = 1.0$). In contrast, among OCX males given a testosterone implant, P35 and P45 males had higher concentrations compared with P75 (*p*s = 0.001 and 0.004, respectively), but did not differ from one another ($p = 1.0$).

Expression of Fos and AVP in the PVN

There was an effect of time-point (post-stress > stress; $F_{1,69} = 21.9$, $p < 0.001$), a near effect of age ($F_{2,69} = 2.9$, $p = 0.064$), and no effect of treatment ($F_{1,69} = 1.1$, $p = 0.290$), on the number of Fos-ir cells in the PVN (see Fig. 3-3B and 3-3D). These effects, however, were obviated by a significant 3-way interaction ($F_{2,69} = 4.9$, $p = 0.010$). Follow-up analyses split by time-point revealed a significant 2-way interaction at baseline ($F_{2,37} = 5.5$, $p = 0.008$) and no effect or interaction of age and treatment 45 min post-stress (ps > 0.1). The difference at baseline was because of greater expression of Fos-ir in testosterone treated P35 males compared with P45 and P75 males that had the same treatment (ps = 0.036 and 0.018, respectively). These difference, however, were largely driven by heightened ir-cell counts in the PVN in two P35 rats (cage partners) and may not be indicative of typical baseline expression.

There was a main effect of treatment on the number of cells expressing AVP-ir in the PVN ($F_{1,72} = 8.6$, $p = 0.004$) with fewer ir-cells in testosterone treated males compared with those with a blank implant, irrespective of age and of time-point (see Fig. 3-3C and 3-3E). There were no main effects or interactions involving age (ps > 0.1) or time-point, although there was a trend for more cells to express AVP 45 min after stress compared with baseline ($F_{1,72} = 3.0$, $p = 0.088$).

Discussion

Consistent with a previous finding (Romeo et al., 2004), we found that the magnitude of the stress response was greater in pre-pubertal adolescent (P35) males compared with adult (P75) males, and the difference is not related to gonadal status. When we administered an equivalent dose of testosterone to males of both age groups, pre-pubertal males continued to have greater circulating concentrations of corticosterone

after restraint stress. We also extended the previous research by demonstrating that testosterone administration to pre-pubertal adolescent males is not capable of dampening the corticosterone response to stress, as it typically does in adulthood (Handa & Weiser, 2014). This does not mean that this age group is unresponsive to testosterone, because we found that testosterone reduced AVP-ir cell counts in the PVN of males at all ages examined. Thus, testosterone can modulate HPA function before puberty, but this is likely region-dependent and produces no effect on corticosterone output.

We included post-pubertal adolescent (P45) males for which there are fewer investigations of HPA function and found that their responses differed from their younger and older comparisons. Post-pubertal adolescent males treated with testosterone had greater post-stress concentrations of corticosterone than did adults, but did not differ from pre-pubertal adolescents. In contrast, post-pubertal adolescents without testosterone replacement responded like adults and had lower concentrations than pre-pubertal adolescents. Therefore, there are developmental shifts in HPA function that occur in the time around puberty and that depend on both the presence and absence of testosterone. That testosterone increased the corticosterone response to restraint stress at this age is contrary to the dampening effect of testosterone that is typically found in adulthood (Goel et al., 2014; Handa & Weiser, 2014). In the present study, the dampening effect of testosterone in adults was not significant, although the difference was in the right direction. The difference may have been significant had adult males received a larger dose; adults had plasma concentrations of ~2 ng/mL, which could be considered a low physiological dose compared to what is typically seen in intact adult males (~4 ng/mL; see Chapter 2, Fig. 2-1A). Individual differences in testosterone in circulation are

associated with the extent of the dampening effect on stress-induced corticosterone release and PVN activation (McCormick & Mahoney, 1999; Viau, Lee, Sampson, & Wu, 2003; Viau & Meaney, 1996).

The basis for the higher corticosterone concentrations in testosterone-treated versus non-treated OCX males at P45 may involve a higher conversion of testosterone to estradiol at this age. A previous study found that, compared with young adults (P68), pre-pubertal juveniles (P28) and post-pubertal adolescents (P48) had more than twice the amount of aromatase activity in the hypothalamus (Lephart & Ojeda, 1990). Moreover, aromatase activity was up-regulated by androgens acting on AR in stress-related brain regions (e.g., bed nucleus of the stria terminalis and mPOA) (Roselli & Resko, 1993), and the androgen-induced increase was greater in pre-pubertal and post-pubertal adolescent males compared with adult males (Lephart & Ojeda, 1990). Greater aromatase activity in the hypothalamus (and possibly other regions) may be critical for sexual differentiation of the brain during adolescence; in early-life, conversion of testosterone to estradiol via aromatase masculinizes the hypothalamus and adult reproductive behaviour and HPA function (Bingham, Gray, Sun, & Viau, 2011; Bingham, Wang, Innala, & Viau, 2012; McCarthy & Konkle, 2005). The mPOA richly expresses aromatase (Tabatadze, Sato, & Woolley, 2014) and undergoes sexual differentiation during adolescence (Ahmed et al., 2008; McCarthy & Konkle, 2005), indicating that it is a target for estrogenic actions at this age. Therefore, pronounced conversion of testosterone to estradiol may account for the increased stress response at P45. Furthermore, androgen regulation of aromatase in pre-pubertal adolescent males provides further evidence that this age group

can respond to androgens in stress-related brain regions, as we saw with AVP expression, despite corticosterone concentrations being unaffected.

Previous studies have found that removal of testicular androgens via OCX increases post-stress expression of AVP heteronuclear (hn) and messenger (m) RNA in the PVN (Seale, Wood, Atkinson, Bate, et al., 2004; Viau et al., 2003), and that peripheral administration of testosterone dampened its expression (Evuarherhe et al., 2009; Seale, Wood, Atkinson, Harbuz, et al., 2004). Moreover, post-stress expression of AVP mRNA in the PVN and resting-state AVP protein in the median eminence (where it is secreted to affect pituitary release of ACTH) were negatively correlated with circulating concentrations of testosterone (Viau et al., 2003; Viau & Meaney, 1996), which indicates that the dampening effect of testosterone on AVP expression is not all-or-none, but depends on the amount in circulation. DHT also reduced post-stress expression of AVP hnRNA in the PVN, but did not affect baseline expression (Lund et al., 2004). Not all findings are consistent; others found no effect of androgen treatment on AVP (Bingaman, Magnuson, Gray, & Handa, 1994; Viau, Chu, Soriano, & Dallman, 1999). Nevertheless, our findings are consistent with the bulk of evidence showing a dampening effect of testosterone on AVP expression in the PVN, which was present at baseline and after 45 minutes of recovery from stress. Additionally, we provide novel evidence that the suppressing effect of testosterone on AVP expression is present in pre-pubertal and post-pubertal adolescents, which suggests AVP is not the basis for age-related differences in the corticosterone response to stress.

As expected, stress increased the expression of Fos in the PVN, as measured 45 minutes after restraint stress compared with males taken directly from the home-cage.

Although we did not observe a significant effect of age ($p = 0.064$), our data was in the same direction as past studies that found pre-pubertal adolescents had more Fos expression in the PVN compared with adults (Lui et al., 2012; Romeo et al., 2006; Viau et al., 2005). Additionally, we did not observe a dampening effect of testosterone, which we expected based on previous findings in adults showing that OCX increased its expression after stress and that post-stress Fos expression was negatively correlated with testosterone in intact males (Viau et al., 2003). In adult males, the dampening effect of testosterone seems to involve actions on AR in the mPOA; direct implants of testosterone reduced expression of *c-fos* mRNA and Fos protein in the PVN, and this effect was reversed with hydroxyflutamide (an AR antagonist) (Williamson et al., 2010). Moreover, lesions to the mPOA block the inhibitory effect of testosterone on PVN Fos expression (Williamson & Viau, 2008). DHT treatment also reduced post-stress expression of *c-fos* mRNA in the PVN of OCX males, whether it was administered peripherally (Lund et al., 2004) or directly above the PVN (Lund et al., 2006). The lack of an effect of testosterone or of age in the present study may be related to the fact that, like corticosterone, the two factors tended to interact on post-stress expression of Fos in the PVN. Unlike corticosterone concentrations, however, there was considerable variability in Fos expression within each group, which may have obfuscated effects.

Taken together, the results of experiment 1 indicate that developmental changes in gonadal status (i.e., testosterone concentrations) do not explain age-related differences in HPA function. Additionally, testosterone's regulation of HPA function changes across the adolescent period and into adulthood, as evidenced by differential effects on the release of corticosterone. Nevertheless, males at each age were responsive to testosterone

in a region- and marker-dependent manner, which leads us to hypothesize that testosterone is being differentially converted to various metabolites at each age. Therefore, in a second experiment, we tested this hypothesis to explain age-related differences in testosterone's regulation of the corticosterone response to stress.

Experiment 2: Antagonism of receptor targets of androgens and effects of HPA stress responses in pre-pubertal and post-pubertal adolescent and adult male rats.

In experiment 2, we sought to replicate the age-specific effects of testosterone on the corticosterone response to stress found in experiment 1. Further, we included DHT-treated males, because unlike testosterone, DHT cannot be converted to estradiol. We also utilized AR and ER antagonists to identify the receptor targets of androgens at each age to explain the findings from experiment 1. We tested the hypothesis that the higher stress-induced corticosterone release in testosterone-treated P45 males involves actions at ER receptors, if testosterone is undergoing greater conversion to estradiol to act on ERα. Because DHT has been shown to suppress HPA responses through actions on AR or via conversion to 3β-diol and actions on ERβ in adults (Handa, Sharma, & Uht, 2011), application of AR or ER antagonists should attenuate the effect of DHT. We had no predictions for ER and AR antagonism in pre-pubertal adolescents.

Methods

Animals

Male Long Evans rats arrived at P23, P33, or P63 (n = 56 per age group) from Charles River (St. Constant, Quebec) in four batches, which were balanced across ages. Animals were housed in same age, same condition (described below) pairs, given free access to food and water, and kept on a 12 hour light-dark cycle (lights on at 10:00). All

procedures were approved by the Brock University Animal Care and Use Committee and were in keeping with the National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) as well as the Canadian Council on Animal Care guidelines.

Orchiectomy, hormone replacement, and receptor antagonism

After 5 days of acclimation to the colony, all males underwent an orchiectomy (OCX) and received a silastic tube implant (S.C.; 1.57 mm i.d., 3.18 mm o.d.; Dow Corning) that contained testosterone (2 cm length; ~10 mg) or DHT (1 cm length; ~5 mg), while under isoflurane. One day after surgery, males were given daily injections of sesame oil (vehicle group) or flutamide (25 mg/kg) or tamoxifen (1 mg/kg) suspended in sesame oil for 6 days. This dose of flutamide was sufficient to suppress pubertal maturation of the gonads and elevated testosterone concentrations in intact male rats (Shin et al., 2002). The dose and injection regimen of tamoxifen is the same as that used by Lund and colleagues. (2006), which was sufficient to block gonadal hormonal modulation of HPA function in adult males. We also included OCX males given a blank (empty) implant and vehicle injections, which provided a comparison group for those with hormone replacement. Therefore, for each of the 3 ages, there were 7 different conditions with 8 males per group and, because experiments were run in 4 batches, we included 2 males from every age and condition in a batch. Two adult males from the DHT-tamoxifen group were removed from the experiment because they had developed an infection.

Stress and sample collection

On P35, P45, or P75, male rats from each condition underwent 30 min of restraint stress, which began ~ 1 to 3.5 hours into the light-dark cycle. The order of testing was counter balanced across ages and conditions and different size restrainers were used for each age group, which provided limited mobility for all rats. Immediately after restraint, males were rapidly decapitated for the collection of trunk blood into ice-chilled glass tubes containing EDTA and were centrifuged at 3000 RCF for 10 min for the collection of plasma, which was frozen at – 20˚C until we performed steroid extraction and hormone assays.

Hormone assays

Steroids were extracted from plasma (100 µL) using diethyl ether and the samples were reconstituted in buffer provided in the enzyme-linked immunosorbent assay kits for the detection of corticosterone and progesterone (Neogen, Lansing, MI). Assay procedures were conducted as per kit instructions and using a Biotech Synergy plate reader. All samples were run on the same day and sensitivity was 0.05 and 0.4 ng / mL for corticosterone, and progesterone, respectively.

Statistical analyses

Because we found no effect of tamoxifen on any measures, we cannot be certain that our manipulation was effective and thus, we excluded the tamoxifen groups from subsequent analyses. Exclusion of the tamoxifen groups did not change the results and simplified the presentation of the data. We used 2 factor (treatment, age) ANOVAs to compare OCX males that received empty implants (and vehicle) to those given testosterone (or DHT) along with vehicle or flutamide. Alpha was set at $p < 0.05$ and post hoc tests were Bonferroni-corrected.

Results

Corticosterone

We compared OCX males without replacement to those given testosterone and vehicle or testosterone and flutamide. There was no effect of treatment ($F_{2,62}$ = 1.3, p = 0.279), no effect of age ($F_{2,62}$ = 1.6, p = 0.213), nor an interaction of the two factors ($F_{4,62}$ = 0.9, p = 0.462). Nevertheless, visual examination of the data (see Fig. 3-3A) reveals that age-specific effects of testosterone were in the same direction as experiment 1 results (see Fig. 3-1A).

When we compared OCX males to those given DHT and vehicle or flutamide, we found no effect of condition ($F_{2,62}$ = 0.8, p = 0.453) or of age ($F_{2,62}$ = 1.4, p = 0.257), nor an interaction of the two factors ($F_{4,62}$ = 0.2, p = 0.930).

Progesterone

When we compared OCX males to those given testosterone and vehicle or flutamide on post-stress concentrations of progesterone, we found no effect of age ($F_{2,62}$ = 1.4, p = 0.246), and an effect of condition ($F_{2,62}$ = 4.5, p = 0.014) that did not depend on age ($F_{4,62}$ = 0.8, p = 0.510). OCX males without hormone replacement did not differ from those given testosterone and vehicle (p = 0.110) or testosterone and flutamide (p = 1.0). Among those given testosterone, flutamide increased the progesterone response to stress relative to vehicle treatment (p = 0.013).

When we compared OCX males without hormone replacement to those that given DHT and vehicle or flutamide, we found an effect of condition ($F_{2,62}$ = 5.2, p = 0.008), but not of age ($F_{2,62}$ = 1.6, p = 0.202), and no interaction of the two factors ($F_{4,62}$ = 1.6, p = 0.187). DHT reduced progesterone relative to those without hormone replacement (p =

0.016) and flutamide fully reversed the effect of DHT ($p = 0.028$). Those without replacement did not differ from those that got DHT and flutamide ($p = 1.0$).

General Discussion

In experiment 1, we found that age-related differences in the corticosterone response to stress were not explained by changes in gonadal status; when pre-pubertal adolescent males were given testosterone, they continued to respond more strongly than did adults (see Fig. 3-1C, middle panel). Moreover, we found that testosterone had no effect on post-stress concentrations of corticosterone in pre-pubertal adolescents, increased concentrations in peri-pubertal adolescents, and moderately reduced concentrations in adults compared with non-replaced OCX males. In experiment 2, we sought to replicate this finding and found evidence for the same pattern of results, although this time the differences failed to reach statistical significance (see Fig. 3-3A).

A potential source of variation in experiment 2 may be related to the repeated daily injections (6x) that males received prior to stress exposure. To examine the receptor targets of androgens, rats were repeatedly injected with AR or ER antagonists and measured post-stress corticosterone and progesterone concentrations. This protocol was like that used by Lund, Hinds, and Handa (2006), who characterized the contribution of various receptor targets on modulating HPA function in adult males. In the present study, however, it may be that repeated handling and injections added additional stressors, and thus the corticosterone results reflect the history of chronic stress rather than acute stress.

Prior stress history is known to influence HPA responses to stress. In response to a homotypic stressor (i.e., a stressor that has been repeatedly experienced in the past), adults will release less ACTH and corticosterone compared with those that experience the

stressor for the first time, providing evidence of habituation (Lui et al., 2011; Romeo, 2010). In contrast, when confronted with a heterotypic stressor (a new stressor), adults often respond more strongly than those that experience the same stressor for the first time (Grissom & Bhatnagar, 2009).

Age-related differences exist, however, regarding how stress history affects HPA responses. Pre-pubertal adolescents do not habituate and may become sensitized to a homotypic stressor, responding as strongly or stronger than those who undergo the stressor for the first time {Romeo et al., 2006; Lui et al., 2011). Importantly, activation of the PVN is suppressed in response to a homotypic stressor at both ages, although the age-related difference still remains (Lui et al., 2011), which suggests that adolescent sensitization may relate to changes in the pituitary and/or adrenals. In response to heterotypic stressors, the age-related difference in corticosterone release was partially mitigated (Lui et al., 2012), which may explain why we failed to see a significant effect of age in experiment 2. Less is known about how gonadal hormones modulate responses to homotypic and heterotypic stressors, but the present results suggest that the effects of androgens on HPA responses are mitigated when the stressor is heterotypic in nature.

We predicted that tamoxifen would reduce the corticosterone response to stress in P45 males given testosterone, because we hypothesized that the pronounced stress response seen in experiment 1 was related to greater conversion of testosterone to estradiol. We found no effect of tamoxifen at any age, however, which led us to drop these groups from the analyses. This finding is in contrast with previous studies that showed that tamoxifen partially blocked the dampening effect of DHT on HPA measures after stress in adulthood (Lund et al., 2006). In the present study, we also failed to see a

dampening effect of DHT on corticosterone, which has been previously demonstrated following central or peripheral administration in adult rats and mice (Handa, Kudwa, Donner, McGivern, & Brown, 2013; Lund, Munson, Haldy, & Handa, 2004a, 2004b). This led us to believe that the dose of DHT that we administered was not sufficient to reduce HPA function, but when we examined progesterone, DHT significantly reduced concentrations in males of all ages. Therefore, either DHT can regulate progesterone, but not corticosterone at the dose given, or this may be further evidence that repeated injection stress masked the effects of gonadal hormones on the corticosterone response to stress.

The same effect was observed with testosterone treatment, whereby progesterone concentrations were reduced compared with no testosterone treatment groups ($p = 0.1$), particularly at P45 and P75, but corticosterone concentrations remained less affected. In both DHT- and testosterone-treated males, flutamide increased post-stress concentrations of progesterone, which indicates androgen-induced suppression of progesterone involves actions at AR. The dampening effect of androgens on progesterone release conflicts with a previous report that corticosterone release in response to acute stress is reduced after OCX in pre-pubertal and adult males (Kalil, Leite, Carvalho-Lima, & Anselmo-Franci, 2013; Romeo, Bellani, & McEwen, 2005). Our results showing that androgens decreased progesterone but not corticosterone after exposure to a heterotypic stressor point to the adrenals as a target for androgenic effects, possibly by altering the expression of enzymes involved in steroidogenesis. We (in Chapter 2) and others (Hueston & Deak, 2014) have previously shown that release of the two hormones in response to stress are highly

correlated, but there may be bias away from corticosterone synthesis after repeated exposure to stressors that renders differences in progesterone more prominent.

As mentioned, a potential limitation of the present study is the repeated injection stress that males underwent before measuring their hormonal responses to restraint. Moreover, there may have been an effect of shipment or OCX surgery that interacted with age, which further compounded the stress history of the animals. Future studies should minimize the stress history of the animals by comparing the effects of age and treatment in those born within the colony and following fewer, less stressful manipulations, possibly by administering antagonists via implants rather than repeated injection. It would also be advantageous to examine other markers of HPA function to determine the sites of androgenic effects in males of different ages. We suspect that age-related differences in gonadal regulation of HPA function may involve actions in peripheral and central regions where aromatase and 5α-reductase are expressed.

Taken together, we have shown that developmental changes in gonadal status does not account for age-related differences in HPA function. Moreover, we have provided evidence of changes in gonadal regulation of HPA function throughout the adolescent period and into adulthood, which were region specific and may involve differential steroid metabolism in the brain and periphery. Findings from the present study and from past research indicate that androgens can regulate certain HPA markers (AVP) and enzymatic activity before puberty (Lephart & Ojeda, 1990), indicating that this age group is sensitive gonadal hormones. More research is required to tease apart the mechanisms that mediate changes in gonadal regulation of HPA markers during this period.

Figures

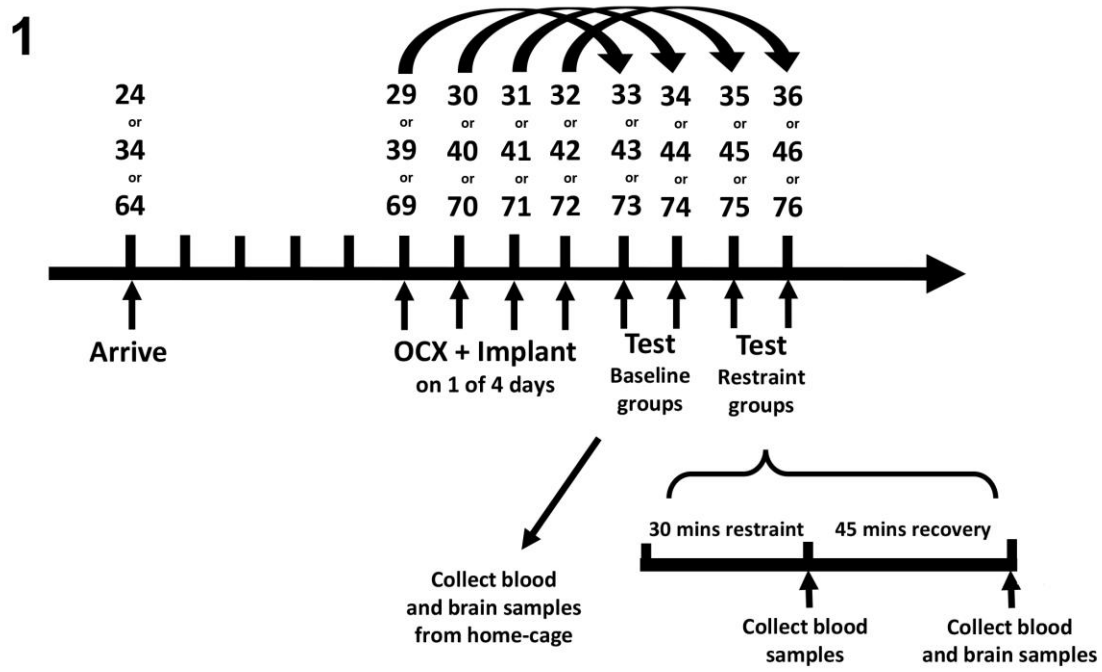

Figure 3-1. Experimental time-table for experiment 1. Male rats of different ages were OCXed and administered an implant that contained testosterone or was left blank on one of four days. Four days after surgery, rats were tested, which involved the collection of blood and brains either directly from the home-cage (Baseline groups) or after exposure to restraint stress (Restraint groups).

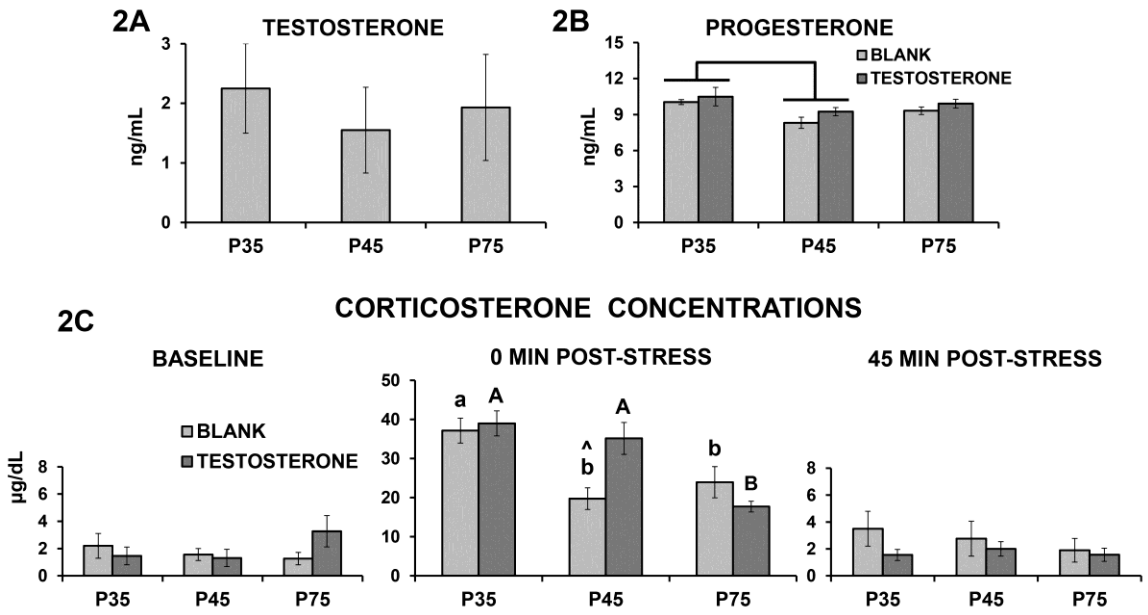

Figure 3-2. Mean (± S.E.M.) concentrations of testosterone (A), progesterone (B), and corticosterone (C). (A) baseline concentrations of testosterone in OCX males of various ages (P35, P45, P75) that received a testosterone implant. (B) baseline concentrations of progesterone in OCX males of different ages that received a blank implant (light grey) or a testosterone implant (dark grey). (C) concentrations of corticosterone at baseline (left panel), 0 min post-stress (middle panel), and 45 mins post-stress (right panel) in OCX males of different ages that received a blank implant (light grey) or a testosterone implant (dark grey). Different lower-case letters indicate an age-related difference in corticosterone among those that received a blank implant. Different upper-case letters indicate an age-related difference in corticosterone among those that received a testosterone implant. ^ indicates a significant effect of treatment (blank vs testosterone) within an age group.

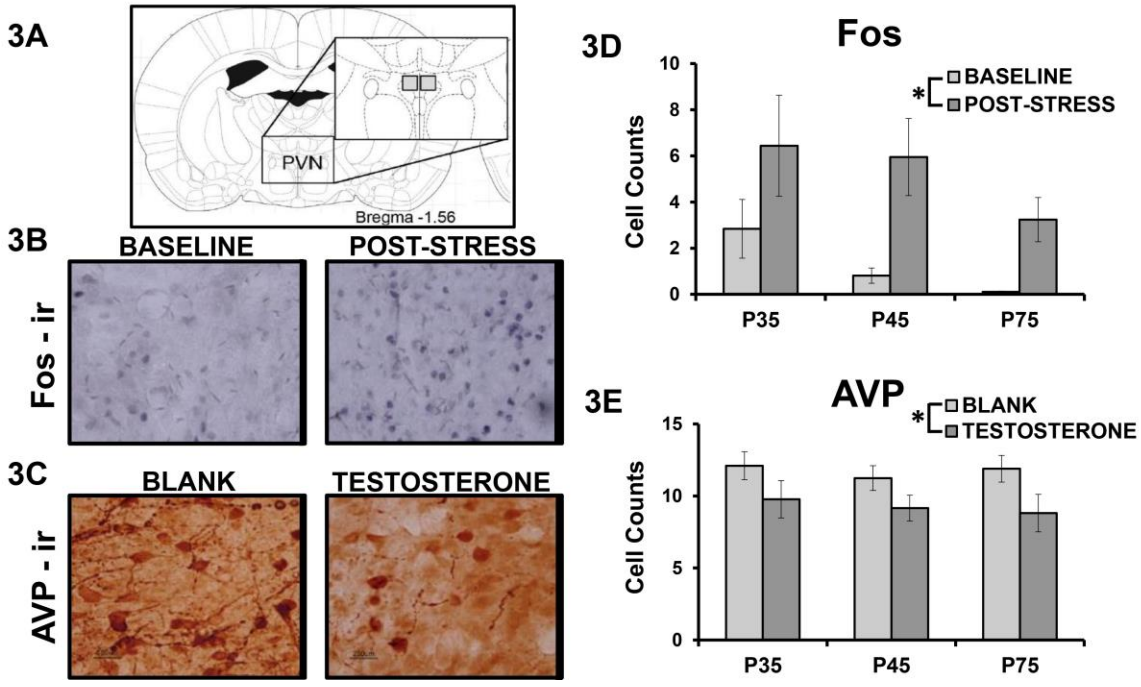

Figure 3-3. Representative images of the medial parvocellular PVN (A) stained for Fos (B) or for AVP (C). Mean (± S.E.M.) number of cells expressing Fos-ir in the PVN (D) in OCX males of different ages (P35, P45, P75) at baseline (light grey) or 45 mins after restraint stress (dark grey). * indicates a significant effect of time-point. Mean (± S.E.M.) number of cells expressing AVP-ir in the PVN (E) in OCX males of different ages that received a blank implant (light grey) or a testosterone implant (dark grey) * indicated a significant effect of testosterone treatment.

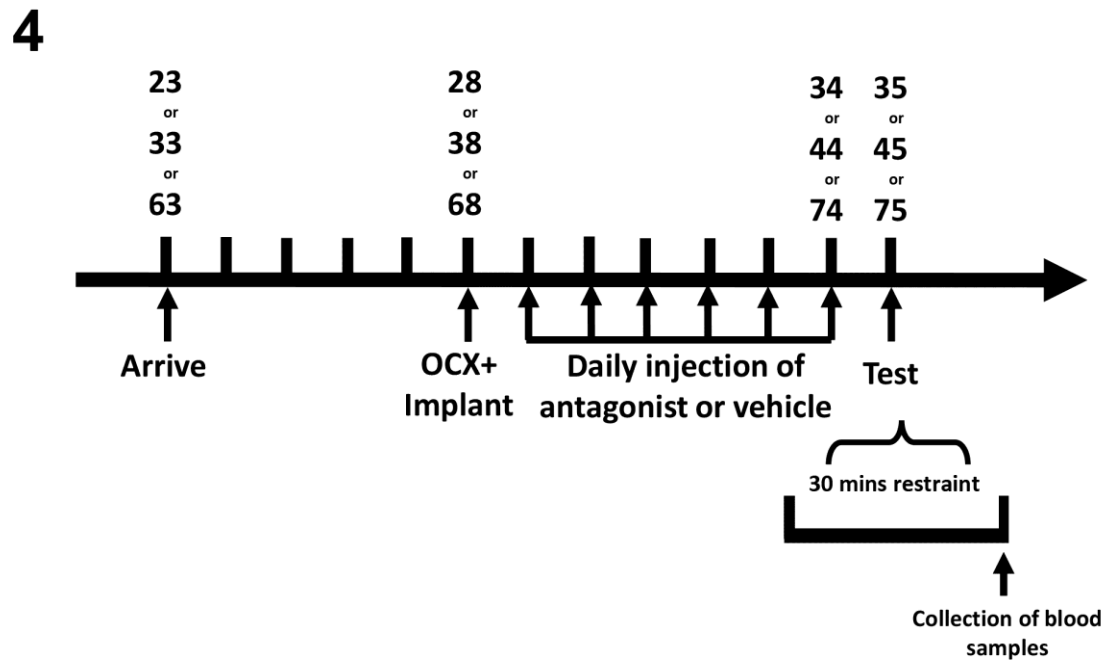

Figure 3-4. Experimental timetable for experiment 2. Male rats at different ages were OCXed and given an implant of testosterone or DHT or one that was left empty. The day after surgery, males were given 6 daily injections of vehicle, tamoxifen, or flutamide, and the next day blood samples were collected immediately after 30 minutes of restraint stress.

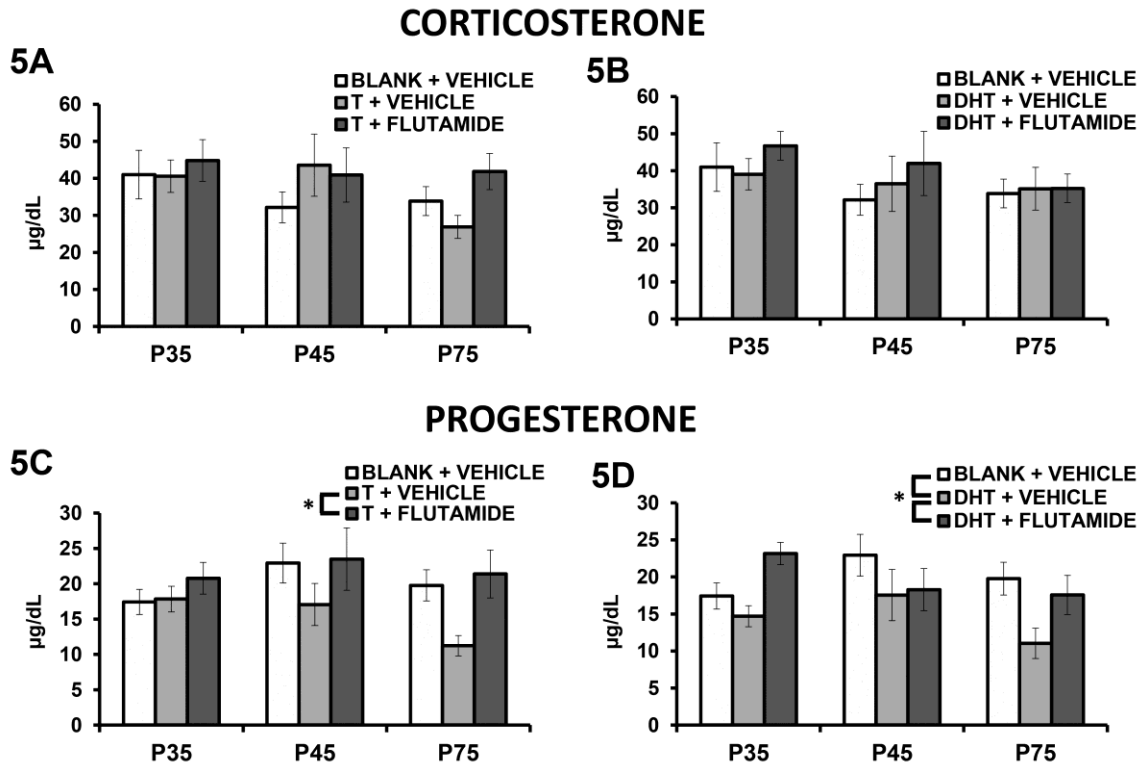

Figure 3-5. Mean (± S.E.M.) concentrations of corticosterone immediately after restraint stress in males of different ages (P35, P45, P75) that received a blank implant versus those that got a testosterone implant (A) or a DHT implant (B) along with injections of vehicle or flutamide. Mean (± S.E.M.) concentrations of progesterone immediately after restraint stress in males that received a blank implant versus those that got a testosterone implant (C) or a DHT implant (D) along with injections of vehicle or flutamide.

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Rationale for Chapter 4

In the previous chapters, I demonstrated marked shifts in neuroendocrine response to stress across the adolescent period and showed that age-related differences are not likely related to GR mediated feedback in the hippocampus or to differences in GR co-chaperones. Moreover, I provided evidence that age-related differences are not mediated by gonadal status and that gonadal hormones influence HPA responses differently before, during, and after puberty. These experiments involved male rats only, and it is important to understand how ovarian hormones regulate HPA function at different ages. As a first step towards such a goal, in this chapter, I investigated gonadal regulation of HPA responses in adult female rats. Previous studies have revealed that estradiol increases stress reactivity and delays the return of corticosterone to baseline (Green & McCormick, 2016), but the findings are not always consistent and variability has been attributed to the duration and dosage of estradiol replacement. Therefore, I used a combination of treatment regimens to examine the effects of ovarian hormones on stressor-induced hormonal responses (corticosterone and progesterone) and to investigate potential mechanisms underlying their effects. Evidence that estradiol slows stress recovery and produces an anti-translocation profile of mRNA expression of GR co-chaperones, *in vitro*, led me to investigate similar measures that were included in chapter 2. Specifically, we measured GR translocation in response to acute stress and mRNA expression of co-chaperones and co-activators, which can modulate GR's transcriptional activity, in the hippocampus. The hippocampus was selected based on its role in HPA negative feedback.

Chapter 4: The effects of ovarian hormones on stressor-induced hormonal responses, glucocorticoid receptor expression and translocation, and genes related to receptor signaling in adult female rats.

This chapter has been submitted for publication:

Green, M. R., Marcolin, M. L., & McCormick, C. M. (submitted). The effects of ovarian hormones on stressor-induced hormonal responses, glucocorticoid receptor expression and translocation, and genes related to receptor signaling in adult female rats. *Stress.*

Author contribution: I was the primary investigator of this work, responsible for the majority of study design, data collection, data analysis, and writing of the manuscript. I performed hormone assays and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) experiments.

Marina Marcolin helped with the administration of ovarian hormone treatments (30%), sample collection (20%), the subcellular fractionation of samples (50%), hormone assays (40%), and running Western blot experiments (80%). Dr. Cheryl McCormick assisted in study design (50%), sample collection (20%), data analysis (30%), and the editing of the manuscript.

Introduction

The hypothalamic-pituitary-adrenal (HPA) axis regulates the release of glucocorticoids (primarily corticosterone in rats). In response to a stressor, increased HPA signaling leads to greater release of glucocorticoids, which act throughout the body on glucocorticoid and mineralocorticoid receptors (GR and MR, respectively). Increased binding of glucocorticoids at receptor targets alters energy metabolism, immune function, and cognitive processes to cope with the stressor (Sapolsky et al., 2000). Moreover, increased GR binding at each level of the axis and in upstream brain regions (e.g., hippocampus and prefrontal cortex) results in negative feedback, whereby HPA activity is suppressed, facilitating a return to baseline concentrations of glucocorticoids (Herman et al., 2012). Whereas brief and infrequent exposures to glucocorticoids can be adaptive for coping with stressors, too much exposure is associated with 'wear and tear' on the body (e.g., cardiovascular disease and immune dysfunction) and an increased risk for psychopathologies such as depression (Juster et al., 2010; McCormick & Green, 2013).

In adult rodents, females have higher baseline and stressor-induced concentrations of corticosterone in circulation compared with males (Babb et al., 2013; Iwasaki-Sekino et al., 2009; Kitay, 1961; Seale et al., 2004a; Viau et al., 2005). Furthermore, after a stressor, females take longer to return to baseline concentrations than do males, which suggests that females have reduced HPA feedback (Iwasaki-Sekino et al., 2009; Kitay, 1961; Weinstock et al., 1998). These sex differences are attributed foremost to circulating concentrations of gonadal hormones; after ovariectomy, females tend to respond like gonadally-intact males, and estradiol replacement potentiates HPA activity and reduces negative feedback (Burgess & Handa, 1992; Kalil et al., 2013; Kitay, 1963; Lunga & Herbert, 2004; McCormick et al., 2002; Seale et al., 2004b; Viau & Meaney, 1991;

Weiser & Handa, 2009; Young, 1996). The stimulatory effects of estradiol are also observed in cycling females, which have more pronounced and prolonged stress responses during proestrus when concentrations of circulating estradiol are highest, compared with other phases of the cycle (Atkinson & Waddell, 1997; Carey et al., 1995; Viau & Meaney, 1991). Estradiol may reduce negative feedback by altering the expression of corticosteroid receptors. For example, estradiol decreased the expression of *GR* mRNA in the hypothalamus and hippocampus of ovariectomized rats (Burgess & Handa, 1993; Peiffer et al., 1991; Seale et al., 2004b). Moreover, ovariectomy increased the number of cytosolic binding sites for dexamethasone in the hippocampus, hypothalamus, and pituitary of adrenalectomized rats (Turner, 1990; Turner & Weaver, 1985). Other studies, however, found no effect of estradiol on binding in stress-related brain regions (Burgess & Handa, 1992; Ferrini et al., 1990).

The dampening effects of estradiol on HPA feedback may extend beyond the number of available corticosteroid receptors to factors that modulate their assembly and activity. In support of this hypothesis, administration of a GR agonist reduced *GR* mRNA expression in the hippocampus of ovariectomized rats, but not in females given estradiol replacement, indicating that estradiol interferes with GR autoregulation (Burgess & Handa, 1992, 1993). A mechanism by which estradiol may alter GR signaling is by reducing GR's transcriptional activity via dephosphorylation (Zhang et al., 2009) or changing the expression of enhancers/repressors (e.g., SRC-1) (Mitev et al., 2003). There is also evidence that ovarian hormones affect the localization of GR; ovariectomized rats had more nuclear GR in the hippocampus than did sham controls when tested at baseline (Sheng et al., 2003). Another study found that estradiol produced an anti-translocation

profile in hippocampal cell culture; mRNA expression of *Fkbp5* and *Ppid* were up- and down- regulated by estradiol, respectively (Malviya et al., 2013). *Fkbp5* codes for the FK506-binding protein 51 (Fkbp51), which reduces both GR's affinity for its ligands and its ability to translocate to the nucleus, whereas Ppid protein promotes translocation (Binder, 2009; Ratajczak et al., 2009; Wochnik et al., 2005). Nevertheless, no studies have examined the effects of ovariectomy or of ovarian hormone replacement on GR localization changes in response to an acute stressor, nor has their effects on co-chaperones been investigated in vivo.

To characterize the actions of estradiol on HPA function and GR signaling, we investigated its effects on plasma corticosterone, GR translocation and the expression of genes that code for corticosteroid receptors, co-chaperones, and a co-activator. We administered estradiol alone or in combination with progesterone to ovariectomized females and measured protein and mRNA expression in the hippocampus because of its involvement in negative feedback (Herman et al., 2012). Progesterone treatment was included because it was found to mitigate the effects of estradiol on HPA activity and feedback (Viau & Meaney, 1991) and can affect corticosteroid receptor binding (Carey et al., 1995). We hypothesized that estradiol, alone or in combination with progesterone, would increase baseline and post-stress corticosterone, consistent with past studies, and reduce expression of cytosolic GR in the hippocampus as well as stressor-induced GR translocation. Further, we hypothesized that baseline mRNA expression of corticosteroid receptor would be concomitantly reduced, whereas genes coding for anti-translocation (e.g., *Fkbp5* and *Bag1*) and pro-translocation (*Fkbp4*) co-chaperones may be augmented and dampened by estradiol treatment, respectively.

Methods

Experiment 1: Effects of ovarian hormones on HPA responses and GR translocation

Animals

Adult female Long-Evans rats (*N* = 100) were obtained from Charles River at approximately postnatal day 60 (P60), housed in same treatment pairs (described below), and were acclimated to the colony for one week. Rats remained gonadally-intact (non-ovariectomized [OVX], *n* = 20) or were bilaterally OVX by the supplier (*n* = 80). All rats were maintained on a 12-hour light-dark cycle (lights on at 0900) with food and water available ad libitum. Procedures were in keeping with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1996) and the Canadian Council on Animal Care guidelines, and were approved by the Brock University Animal Care and Use Committee.

Experimental manipulations and sample collection

Although all females were obtained at the same time, they were tested in batches and were 1-2 months post-OVX at the time. Females were assigned randomly to one of five treatments (*n* = 20 per treatment). OVX females received four days of daily subcutaneous injections of vehicle (VEH), estradiol benzoate (EB, 25 µg/kg in ~250 µl of sesame oil vehicle, dosing regimen based on [Weiser & Handa, 2009]), or EB and progesterone (EB+P) (P: 0.5 mg in 200 µl of sesame oil vehicle, based on [McCormick & Singh, 1996]), and one group received vehicle on days 1 and 3, EB on day 2, and P on day 4 (EB+P [FD]; fewer doses), which was done to better match the hormonal profile of naturally cycling female. The EB+P(FD) treatment regimen is comparable to that used to produce behavioral estrus in OVX rats (McCormick et al., 2013). The non-OVX females

received a daily vehicle injection to control for any effects of handling and injection stress. Injections before the test day were given during the lights on phase (between 1100 and 1700) and, on the day of testing, injections and sample collection occurred from 0800-0900 and from 1100-1200, respectively (restraint stress started as early as 10:30).

Trunk blood and brain tissue was collected via rapid decapitation without anesthetic either directly from the home cage (baseline) or after 30 minutes of restraint (post-stress) in Plexiglas® restrainers (n = 10 per group). Blood samples were collected in ice-chilled glass tubes containing EDTA and were centrifuged at 4˚C (3000 RCF for 15 min) for the collection of plasma, which was kept at -20˚C until assayed. Brains were extracted rapidly, sliced into 1 mm thick coronal sections, and placed onto ice-chilled slides, which were immediately frozen on dry-ice. Brain sections were stored at -80˚C until tissue processing.

Hormone assay

Corticosterone and progesterone steroids were extracted from baseline and post-stress plasma samples using diethyl ether and reconstituted in buffer provided in the enzyme-linked immunosorbent assay kits (Neogen, Lansing MI), which were run according to the kit instructions and quantified using a Biotech Synergy plate reader. Assay sensitivity was 0.05 ng/mL for corticosterone and 0.4 ng/mL for progesterone. Intra- and inter-assay variabilities were < 5% and 12%, respectively for both hormones.

Protein extraction

To determine GR expression in the cytosolic and nuclear compartments, the whole hippocampus was dissected on dry-ice using a tissue puncher (1 mm inner diameter) and tissue from two females of the same treatment and stress condition were

pooled together (*n* = 5 per group). Throughout the protein extraction procedure, samples were kept on ice or at 4˚C. An appropriate volume of cold S1 buffer (10mM HEPES pH 7.5, 1.5mM MgCl2, 10mM KCl, 2mM DDT, 1mM EDTA, 1mM EGTA, 0.5% Triton-X) was added to the samples, which were homogenized in a Bullet Blender® and centrifuged (17,200 RCF for 3 min). The pellet was retained for preparation of the nuclear fraction, and the supernatant was collected and re-centrifuged (16,000 RCF for 20 min) and the resulting supernatant was retained as the final cytosolic fraction. To wash the nuclear pellets, samples were re-suspended in the same volume of S1 buffer using a 26.5-gauge needle, vortexed, and centrifuged (17,200 RCF for 3 min). The supernatant was discarded and the pellets were re-suspended and homogenized in ~1.2 pellet volumes of cold S2 buffer (20mM HEPES pH 7.5, 400mM NaCl, 1.5mM MgCl2, 0.1mM EDTA, 20% glycerol, 2mM DTT), then incubated on ice for 1 hour during which time the samples were vortexed for 10 seconds every 10 minutes. After the incubation, the samples were centrifuged (17,200 RCF for 30 minutes) and the supernatant was retained as the final nuclear fraction. S1 and S2 buffer included a protease inhibitor cocktail (Sigma, 5892791001). Protein concentrations were determined for cytosolic and nuclear fractions using the Bio-Rad Protein Assay procedure (Bio-Rad) and a Biotech Synergy plate reader. Samples were brought to an equal concentration using the appropriate buffer and an equal volume of 2X Lammaeli buffer was added. Afterwards, samples were heated at 70˚C for 5 minutes, centrifuged (16,000 RCF for 10 min), and then stored at -20˚C until Western blot experiments were conducted.

Western blotting

To measure GR translocation, cytosolic and nuclear samples (30 µg of protein each) were resolved on 6% acrylamide gels and transferred to a PVDF membrane. Total protein was measured via Ponceau stain and used to normalize GR values. Membranes were reactivated, washed (3 X 5 min) in Tris-buffered saline with 1% Tween 20 (TBS-T), and blocked for 1 hour with 5% non-fat milk powder dissolved in TBS-T. Membranes incubated overnight at 4˚C in TBS-T containing 1% milk block and rabbit anti-GR (M20, Santa Cruz; 1:500 and 1:200 for cytosolic and nuclear samples, respectively). Next day, membranes were washed and then incubated for 45 minutes at room temperature in TBS-T containing 1% milk block and goat anti-rabbit (Life Technologies, AlexaFluor 488; 1:5000), washed again, and then were imaged using a Bio-Rad VersaDoc (MP 4000). Samples from each condition and stress condition (i.e., baseline or post-stress) were counterbalanced within and across gels.

To validate the subcellular fractionation protocol, cytosolic and nuclear samples (10 µg of protein each) were resolved on a 12% acrylamide gels, blocked with 5% BSA, and probed with rabbit anti-histone H3 (D1H2; Cell Signaling; 1:1,000) and rabbit anti-GAPDH (14C10; Cell Signaling; 1:1,000) in TBS-T with 1% BSA.

Experiment 2: Effects of ovarian hormones on HPA responses and gene expression

Animals

Female Long-Evans rats (N = 48) were OVX by the supplier (Charles River) and arrived approximately 9 days later at P60 +/- 2 days. Rats were housed in same-treatment pairs (described below), kept on a 12-hour light-dark cycle (lights on at 0900), and were given free access to food and water. All procedures were in keeping with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-

23, revised 1996) and the Canadian Council on Animal Care guidelines, and were approved by the Brock University Animal Care and Use Committee.

Experimental manipulations and sample collection

Seven days after the OVX females arrived, they were randomly assigned to one of three treatments (n = 16 per treatment), which involved four days of daily subcutaneous injections of vehicle (VEH), estradiol benzoate (EB), or EB and progesterone (EB+P); doses were the same as used in Experiment 1. Thus, females had been ovariectomized for 20 days at the time of testing. Experiment 2 did not include non-OVX and OVX-EB+P(FD) females, because these groups did not differ significantly from OVX-EB+P on corticosterone concentrations in Experiment 1. Injections before the test day were given during the lights on phase (between 1100 and 1700) and, on the day of testing, injections and sample collection occurred from 0800-0900 and from 1100-1400, respectively (stress started as early as 10:30).

Half of the females in each treatment group were rapidly decapitated directly from the home cage (baseline) for the collection of trunk blood and brain tissue, as in Experiment 1. The other half of the animals underwent 30 minutes of restraint in Plexiglas® restrainers. Blood was collected from these females immediately (0 min), 45 min, and 90 min after the 30 minutes of restraint stress ended (first two samples from the tail vein, 90 min sample from trunk blood; brains also collected); rats were returned to home cages between collections. The first tail vein sample (0 min post-stress) was obtained by nicking the end of the tail (~1-2 mm) using scissors, and the second sample (45 min post-stress) was obtained by dislodging the scab that formed after the tail nick (handling and collection of tail vein samples occurred within 2 minutes of removal from

the restrainer and the home-cage for the first and second post-stress collection time-points, respectively). Blood was collected into ice-chilled tubes containing EDTA, centrifuged at 4˚C (3,000 RCF for 10 minutes), and plasma was stored until assayed (see methods Experiment 1). These time-points were selected based on previous evidence that adult OVX females have intermediate concentrations of corticosterone 45 minutes after termination of the stressor compared with baseline and post-stress concentrations, which reflects partial recovery at this time-point (Romeo et al., 2004).

RT-qPCR

Approximately 6-8 punches (1 mm inner diameter) were collected on dry-ice from the ventral hippocampus and total RNA was extracted using a Fatty Tissue RNA Purification Kit (Norgen) as per kit instructions. RNA concentration and quality was assessed using a Nanodrop and sample (500 ng of RNA) were reverse transcribed using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Primers were designed to measure mRNA expression for genes of interest (see Table 1). Samples were run in triplicate using a CFX Connect Real-Time System and SYBR green (Bio-Rad Laboratories) and were counterbalanced within and across plates. Gene expression was normalized to ubiquitin C (*Ubc*), hypoxanthine phosphoribosyltransferase 1 (*Hprt1*), and peptidylprolyl isomerase A (*Ppia*), which demonstrated good stability and did not differ based on stress condition or treatment. Three baseline (2 VEH, 1 EB) and three post-stress (2 VEH, 1 EB) brains were compromised during collection and were thus excluded from the gene expression studies (resulting in n = 6-8 per group).

Statistical analyses

For experiment 1, we used between-subjects (5 treatment x 2 stress condition) ANOVAs on concentrations of corticosterone and progesterone and on cytosolic and nuclear GR expression in the hippocampus. For experiment 2, between-subjects (3 treatment x 2 stress condition) ANOVAs were used for hippocampal mRNA expression of various stress-related genes. Baseline corticosterone concentrations were analysed with a one-way (3 treatment) ANOVA. Post-restraint corticosterone concentrations were analysed with a mixed-model (3 treatment x 3 time-point) ANOVA. Significant interactions were followed up with simple effects analyses where appropriate and post-hoc tests were Bonferroni-corrected with statistical significance set at $p < 0.05$.

Results

Experiment 1: Effects of ovarian hormones on HPA responses and GR translocation

Corticosterone

There was a significant effect of treatment ($F_{4,90} = 4.7$, $p = 0.002$) and stress ($F_{1,90} = 335.2$, $p < 0.0001$) on corticosterone concentrations, and no interaction of the two factors ($F_{4,90} = 1.4$, $p = 0.256$). As expected, stress increased corticosterone across all treatments. Moreover, OVX-VEH females had lower concentrations of corticosterone compared with non-OVX females and with OVX-EB, OVX-EB+P, and OVX-EB+P(FD) females (all $p < 0.05$, see Fig. 1A). The lower corticosterone in OVX-VEH females was irrespective of stress condition and consistent with past evidence of a stimulatory effect of estradiol on baseline and post-stress concentrations.

Progesterone

There was a main effect of treatment ($F_{4,90} = 39.2$, $p < 0.001$) and stress ($F_{1,90} = 55.0$, $p < 0.001$) on progesterone concentrations, and an interaction between the two

factors ($F_{4,90}$ = 2.5, p = 0.046). Follow up analyses indicated that concentrations of progesterone were lower at baseline compared with post-stress in all groups (all p < 0.05), except for OVX-EB+P (p = 0.182). In baseline samples, non-OVX females had higher progesterone concentrations than did all other groups (all p < 0.05, see Fig. 1B) except OVX-EB+P (p = 0.999). Moreover, OVX-EB+P females did not differ from OVX-EB+P(FD) (p = 0.660), but they did have higher concentrations than OVX-EB (p = 0.039) and OVX-VEH (p < 0.001) females. OVX-EB+P(FD) also had higher concentrations than did OVX-VEH females (p = 0.007), but did not differ from OVX-EB females (p = 0.999). For post-stress samples, the same treatment differences were observed as at baseline (all p < 0.05), except non-OVX females had higher concentrations of progesterone than did all other groups (all p < 0.05).

GR translocation

There was a significant effect of treatment ($F_{4,38}$ = 3.2, p = 0.022) on cytosolic GR expression in the hippocampus, but no effect of stress ($F_{1,38}$ = 1.2, p = 0.277), nor an interaction between the two factors ($F_{4,38}$ = 1.7, p = 0.178). Post hoc tests indicated that OVX-VEH treated females had more cytosolic GR than OVX-EB and OVX-EB+P treated females (all p < 0.05; Fig. 1C). Although the omnibus interaction was not significant, likely because of reduced power from small group sizes (n = 4-5 per treatment and stress condition) and the 5 x 2 design, separate analyses for each stress condition suggested the effect of treatment was driven by differences at baseline ($F_{4,19}$ = 6.2, p = 0.002) rather than post-stress ($F_{4,19}$ = 0.2, p = 0.941). Moreover, compared with baseline, stress decreased cytosolic GR in OVX-VEH females only (t_8 = 2.7, p = 0.026). There was evidence of GR translocation in all groups on the basis of increased nuclear

GR expression in post-stress compared with baseline samples (main effect of stress, $F_{1,38}$ = 14.3, p = 0.001; see Fig. 1D). The effect of treatment ($F_{4,38}$ = 0.3, p = 0.900) and the interaction of treatment and stress ($F_{4,38}$ = 0.4, p = 0.832) were not significant.

Experiment 2: Effects of ovarian hormones on HPA responses and gene expression

Corticosterone

Although the pattern of differences paralleled those in experiment 1, the effect of treatment on corticosterone concentrations at baseline missed statistical significance ($F_{2,21}$ = 2.7, p = 0.087). For post-stress samples, based on previous studies, we predicted that estradiol treatment would increase corticosterone concentrations in response to stress and would slow the recovery to baseline compared to vehicle treatment, and that progesterone would mitigate the effect of estradiol (Burgess & Handa, 1992; Viau & Meaney, 1991; Weiser & Handa, 2009). There was an effect of treatment ($F_{2,21}$ = 5.8, p = 0.01) and of time-point ($F_{2,42}$ = 221.3, p < 0.0001) on corticosterone concentrations after restraint, as well as an interaction between the two factors ($F_{4,42}$ = 9.6, p < 0.001). Follow-up analyses at each time-point indicated that at 0 min post-stress, OVX-VEH treated females had lower concentrations of corticosterone than did OVX-EB (p < 0.001) and OVX-EB+P (p = 0.034), and OVX-EB+P had lower concentrations than OVX-EB (p = 0.012; see Fig. 2). Thus, progesterone mitigated some of the stimulatory effects of EB. There was no effect of treatment at 45 or 90 min post-restraint (only the higher corticosterone 45 min after restraint in OVX-EB+P females compared with OVX-VEH females was marginally significant, p = 0.053).

Gene expression

Contrary to our predictions, there was no effect of treatment on mRNA expression of *Nr3c1* (*GR*), *Nr3c2* (*MR*), *Ncoa1 (SRC-1), Fkbp5, Fkbp4,* or *Bag1* (all $p > 0.25$; see Fig. 3). Moreover, treatment did not interact with stress on the expression of our genes of interest (all $p > 0.5$). There was, however, a higher expression of *Fkbp5* post-stress compared with baseline that was irrespective of treatment ($F_{2,36} = 4.8$, $p = 0.035$; see Fig 3D).

Discussion

We hypothesized that estradiol would increase HPA activity and that we would see a corresponding reduction in GR expression and activity, which we assessed by measuring cytosolic and nuclear GR in the hippocampus before and after acute restraint stress. Consistent with the literature (Handa & Weiser, 2014) and with our hypothesis, ovariectomy reduced baseline and post-stress concentrations of corticosterone relative to gonadally-intact females, and treatment of estradiol reversed the effect of ovariectomy. Moreover, ovariectomy increased cytosolic GR relative to several estradiol-treated groups, and this group was the only one to have a stress-induced decrease in cytosolic GR, which supported our hypothesis that estradiol restricts GR expression and activity. As expected, stress increased nuclear GR expression, but contrary to our hypothesis we did not see an effect of estradiol treatment. Therefore, we found partial evidence that estradiol reduces feedback by restricting GR expression and activity. Contrary to our hypotheses, there were no differences in the expression of genes that code for corticosteroid receptors or co-chaperones and co-activators known to modulate corticosteroid receptor activity.

Although this study and many others found that estradiol potentiated HPA activity, some studies have yielded contradictory results. For example, some reported no effect of estrous cycle phase on adrenocorticotropic hormones (ACTH) or on corticosterone concentrations after stress (Babb et al., 2013; Rivier, 1999). Others found that estradiol replacement had no effect (Serova et al., 2010) or reduced (Ferreira-Silva et al., 2009) concentrations of corticosterone in ovariectomized rats. The most conflicting results, however, are related to post-stress concentrations of ACTH, with a number of studies reporting increases (Burgess & Handa, 1992; Carey et al., 1995; Leśniewska et al., 1990; Seale et al., 2004b; Viau & Meaney, 1991; Weiser & Handa, 2009) and decreases (Babb et al., 2013; Figueiredo et al., 2007; Larkin et al., 2010; Redei et al., 1994; Young et al., 2001) in rats given estradiol. Discrepancy in results may involve the time of sample collection after stress given that ACTH is released in pulses and is rapidly metabolized after its release (Reith & Neidle, 1981). Additional variability may stem from differences in the time between ovariectomy and treatment, as well as the dosage and duration of hormone treatment. For example, some studies administered estradiol at the time of surgery (Babb et al., 2013; Burgess & Handa, 1992; Seale et al., 2004b), whereas others waited until 6 weeks after ovariectomy (Redei et al., 1994). Further, some studies used doses that exceed those typically found in proestrus (Carey et al., 1995; Viau & Meaney, 1991) or utilized long treatment durations (Burgess & Handa, 1992). One strength of the current study is that we compared stress responses of gonadally-intact females with those of ovariectomized rats that received one of several ovarian replacement regimens, which were delivered over a brief period (4 days). Consistent with

the general literature, we found that estradiol increased the stress response whether it was administered daily or once only 48 hours before sample collection.

Actions of estradiol on HPA activity occur in the periphery as well as in the brain. Estradiol increases adrenal content and weight (Kitay, 1963; Leśniewska et al., 1990; Lunga & Herbert, 2004; McCormick et al., 2002) as well as adrenal sensitivity to ACTH (Atkinson & Waddell, 1997; Figueiredo et al., 2007). Stimulatory effects of estradiol on HPA function are observed when administered to the PVN (Liu et al., 2012; Weiser & Handa, 2009) or to the medial preoptic area (McCormick et al., 2002). When selective estrogen receptor agonists were administered to the paraventricular nucleus (PVN) of the hypothalamus, ERα agonists increased, whereas ERβ agonists decreased, HPA activity (Liu et al., 2012; Weiser & Handa, 2009). Thus, the stimulatory effects of estradiol on HPA activity seem to be mediated by ERα, possibly in GABAergic neurons of the peri-PVN (given that the parvocellular portion is virtually devoid of ERα (Handa & Weiser, 2014; Miller et al., 2004) and in upstream brain regions.

In addition to potentiating HPA activity, there are many reports that estradiol impaired negative feedback as evidenced by delayed recovery after stress and reduced suppression of corticosterone after treatment with a GR agonist (Burgess & Handa, 1992; Carey et al., 1995; Viau & Meaney, 1991; Weiser & Handa, 2009). Estradiol's dampening of HPA feedback also appears to be mediated by ERα, at least near the PVN, and occurs at baseline and after stress (Weiser & Handa, 2009), which means that the stimulatory effects of estradiol in the brain may involve reduced suppression rather than enhanced drive. Little is known, however, regarding the downstream mechanisms of estradiol-induced suppression or its actions in other brain regions important for negative

feedback, but changes in corticosteroid receptors or their activity may be involved. Surprisingly, we found no evidence of estradiol-induced suppression of HPA feedback in the current study, perhaps because samples were obtained at 45 minutes; at an earlier recovery time-point, differences may have emerged. We did, however, find that ovariectomy increased the expression of cytosolic GR in the hippocampus, which may allow for more GR-mediated feedback. The greater baseline densities of cytosolic GR in the hippocampus may be because of the lower baseline corticosterone concentrations in the ovariectomized, non-replaced females compared with the other groups. Although one study showed a trend for ovariectomy to increase GR binding capacity for corticosterone in the hippocampus (Turner & Weaver, 1985), others found no effect of estradiol on binding capacity for GR agonists (Burgess & Handa, 1992; Ferrini & de Nicola, 1991).

Reports that ovariectomy increased nuclear GR (Sheng et al., 2003) and that estradiol treatment produced an anti-translocation profile of co-chaperones in cell culture (Malviya et al., 2013) led us to investigate GR translocation. Consistent with our predictions, only the ovariectomized, non-replaced group showed a stress-induced reduction of cytosolic GR in the hippocampus, which suggests that estradiol may restrict GR translocation. Thus, when we examined nuclear GR, we expected to find a concomitant increase in stress-induced translocation in ovariectomized, non-replaced females compared with the other conditions, but the results indicated a similar change across groups. One possible explanation for why ovariectomized females had greater stress-induced GR translocation than the other groups based on differences in the cytosol and not in the nucleus is that ovariectomy may have promoted GR trafficking to other cellular compartments (e.g., the cell membrane). Although corticosterone treatment

increased membrane-bound GR in a hypothalamic cell line (Deng et al., 2015), it is unclear what role estradiol plays, if any, in affecting GR trafficking to the membrane. Another possibility is that our experiment was sensitive to detect group differences in stress-induced changes in the cytosol and not in the nucleus because GR is predominantly located in the nucleus even at baseline (Sheng et al., 2003). Nevertheless, our study seems to be the first to examine estradiol's effect on GR translocation following acute challenge in vivo, but our translocation experiment involved small group sizes and as such warrants replication.

Estradiol's actions on HPA stress responses may involve mineralocorticoid receptors (MR); for example, one study found that estradiol reduced MR's binding capacity (Carey et al., 1995). Unlike GR, however, MR are mostly bound under basal conditions and thus we did not expect to see prominent stress-induced changes in MR that would be affected by gonadal hormones. Moreover, others have found more evidence for actions of estradiol on GR than on MR (Burgess & Handa, 1992, 1993). It would be worthwhile, however, to investigate phosphorylation of corticosteroid receptors, which can induce translocation and increase transcriptional activity in the absence of ligand-binding (Hapgood et al., 2016). In support of this, a previous study found that estradiol dephosphorylates GR in breast cancer cell lines (Zhang et al., 2009). Therefore, it is unclear whether the stress-induced decrease in cytosolic GR in ovariectomized, non-replaced females reflects greater receptor binding, phosphorylation, or both. Given that ovariectomized females also released less corticosterone than gonadally-intact females and ovariectomized females treated with estradiol alone or in combination with

progesterone, we may have seen a bigger difference had they been treated with a comparable dose of corticosterone.

Based on the greater amount of GR protein in the hippocampus of ovariectomized, non-replaced rats relative to other groups, we expected to see a concomitant increase in corticosteroid receptor mRNA at baseline relative to estradiol replaced rats. Nevertheless, there were no group differences in either *GR* or *MR* mRNA expression at baseline. Previous studies have yielded mixed results with some finding that estradiol treatment reduced *MR*, but not *GR*, in the hippocampus (Carey et al., 1995; Peiffer et al., 1991) and others finding a decrease in both *MR* and *GR* (Burgess & Handa, 1993). Moreover, one study found that, relative to sham-operated controls, ovariectomy reduced *GR*, but not *MR*, mRNA in the hippocampus (Patchev & Almeida, 1996). Therefore, effects of ovariectomy and of estradiol replacement on corticosteroid receptor transcript remain inconsistent and may not be a good measure for actions on receptor protein levels or activity.

The decrease in cytosolic GR post-stress compared with baseline in ovariectomized females, which we interpreted as evidence of translocation, was not accompanied by differences in baseline mRNA expression of co-chaperones (*Fkbp5, Fkbp4, Bag1*) that are known to modulate GR assembly and trafficking. This lack of difference contradicts previous work in hippocampal cell culture that found estradiol produced a GR anti-translocation profile of gene expression (Malviya et al., 2013). Thus, more investigation of protein expression of co-chaperones and their interactions with corticosteroid receptors in vivo is required to elucidate their role, if any, on receptor dynamics and negative feedback. Surprisingly, we did not find that estradiol altered the

effect of acute stress on the expression of any of the genes we investigated. This result conflicts with a previous report that estradiol prevented the reduction in hippocampal *GR* mRNA and protein (as evidenced by binding assay, which could reflect receptor densities and/or changes in their affinity for agonists) that was found in ovariectomized, non-replaced females treated with a GR agonist (Burgess & Handa, 1992, 1993). Although estradiol has been shown to alter the expression of the steroid receptor co-activator, *Ncoa1* (*SRC-1*), in a region-specific manner (Mitev et al., 2003), we did not observe any group differences, consistent with a previous study showing that ovariectomy did not affect its expression in the hippocampus (Zhang et al., 2011).

Although the effects of ovariectomy are attributed to the reduction of circulating estradiol in females, progesterone is also released from the ovaries and may mitigate some of the effects of estradiol on HPA activity (Viau & Meaney, 1991) or have suppressing actions on its own (Figueiredo et al., 2007). Therefore, we examined the effects of progesterone co-treatment with estradiol compared with those that received no replacement or estradiol alone. The results from experiment 2 supported past reports that progesterone partially mitigates estradiol's stimulatory effects. In experiment 1, however, progesterone co-treatment had no effect. Others found that progesterone had no effect on HPA responses to stress (Ferreira-Silva et al., 2009; Young et al., 2001). Thus, it remains unclear whether progesterone treatment buffers the stress response in females and, if so, what mechanisms are involved. One study found that, in adrenalectomized and ovariectomized females given corticosterone replacement, progesterone treatment increased *GR* mRNA in the hippocampus, which may promote negative feedback (Patchev & Almeida, 1996). In the current study, however, progesterone did not alter *GR*

mRNA or protein in this region. We did find, however, that stress increased the release of progesterone, which is consistent with other studies (Kalil et al., 2013; Simone & McCormick, 2017). Moreover, although the difference was not significant (likely because of the number of comparisons), estradiol treatment tended to increase progesterone concentrations relative to ovariectomized females without estradiol, possibly by enhancing synthesis and decreasing degradation (Munabi et al., 1983). Thus, estradiol-induced increases in progesterone may mitigate the direct stimulatory effects of estradiol in gonadally-intact females. Others, however, have found no effect of ovariectomy or estradiol replacement on progesterone release (Kalil et al., 2013).

In summary, our results show that as little as one administration of estradiol 48 h before sample collection can increase stress-induced corticosterone release. Although we provide evidence that ovariectomy increases the amount of cytosolic GR receptors, we found no effect of ovariectomy or of ovarian hormone replacement on changes in nuclear GR expression in response to an acute stressor or on mRNA expression of co-chaperones that are known to modulate ligand binding and receptor trafficking within the hippocampus. Although estradiol's effects on increasing corticosterone release are attributable to actions at ERα (Liu et al., 2012; Weiser & Handa, 2009), the downstream mechanisms for the effects on stress-induced increases and recovery remain to be elucidated.

Tables

Table 4-1. Sequences of primers used in RT-qPCR reactions.

Gene	Forward	Reverse
Nr3c1 (GR)	GTGCTACCCCGACAGAGAAG	CCGCCAAAGGAGAAAGCAAG
Nr3c2 (MR)	CAGCTCACCTCCATTACGCA	CTTCACGACCTGGCTCATCT
Fkbp5	TTCCCTCGAACGCAACTCTC	GTCGTGGTCTTCTCCTTCGC
Fkbp4	GAAGTCGGGGAAGGGGAAAG	GCATGTGGTGGGATCTGGAA
Bag1	TAGCGGAAGAGGTGACCCAG	CTCCTGTGGCCTCTTCAACA
Ncoa1 (SRC-1)	CAGTGGCAAAGATGAGACG	CGAGTATGGCTGGGGATAG
Hprt1	CAGACTTTGCTTTCCTTGG	CCGCTGTCTTTTAGGCTTTG
Ubc	CCAAGAAGGTCAAACAGG	GACACCTCCCCATCAAAC
Ppia	CCGCTGTCTCTTTTCGCC	ATCCTTTCTCCCCAGTGCTC

Figures

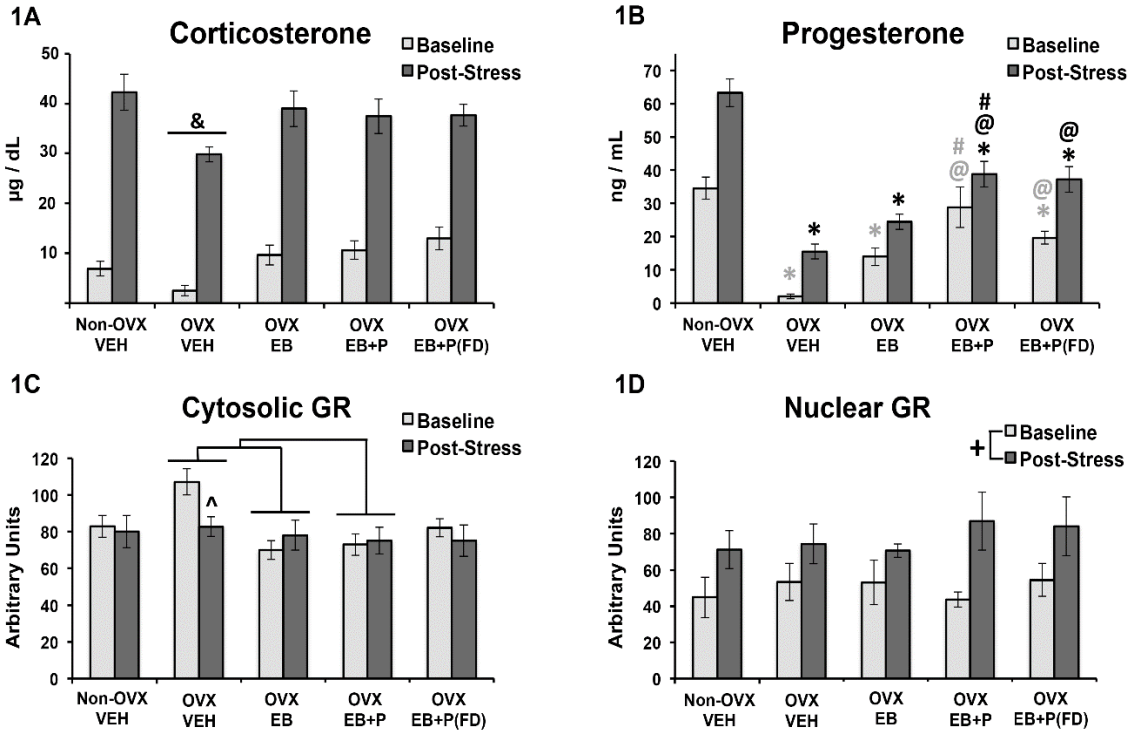

Figure 4-1. Mean (± S.E.M.) plasma concentrations of (A) corticosterone and (B) progesterone and of hippocampal expression of GR in the (C) cytosol and (D) nucleus at baseline (light-grey bars) or post-stress (dark-grey bars) in gonadally-intact (non-ovariectomized [OVX]) females and in OVX females from different treatment groups; OVX females received four daily injections of either vehicle (VEH), estradiol benzoate (EB), EB and progesterone (EB+P), or EB+P (FD; fewer doses), which was done to mimic release in naturally cycling females. (A) & indicates lower than all other treatment groups irrespective of stress condition (all $p < 0.05$ for OVX-VEH compared with all other groups). (B) grey symbols are used for within baseline comparisons of treatment effects and black symbols are used for post-stress comparisons of treatment effects; * indicates when lower than non-OVX females; @ indicates when higher from OVX-VEH female; # indicates when higher than OVX-EB females (only statistically significant

groups differences are pointed out, all $p < 0.05$). (C) ^ indicates a significant difference between baseline and post-stress within a treatment group ($p = 0.026$), and the lines above bars indicate differences between OVX-VEH and OVX-EB or OVX-EB+P (all $p < 0.05$). (D) + indicates an effect of stress irrespective of treatment group ($p = 0.001$).

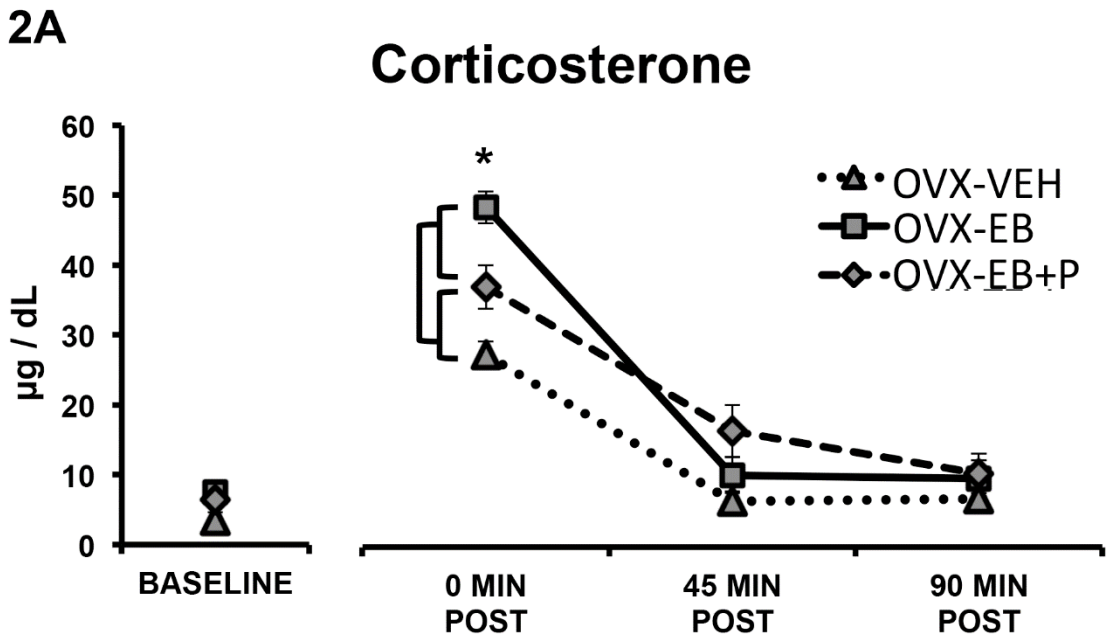

Figure 4-2. Mean (± S.E.M.) plasma concentrations of corticosterone collected straight from the home-cage (baseline) or from repeated sampling taken at 0, 45, and 90 minutes after 30 minutes of restraint stress in ovariectomized (OVX) females from different treatment groups; OVX females received four daily injections of vehicle (VEH), estradiol benzoate (EB), or EB and progesterone (EB+P). * indicates a significant effect of treatment within that time-point and the bars indicate which groups differ; at 0 mins post-stress, OVX-VEH had lower concentrations than OVX-EB+P ($p = 0.034$) and OVX-EB ($p < 0.001$), and OVX-EB+P had lower concentrations than OVX-EB ($p = 0.012$).

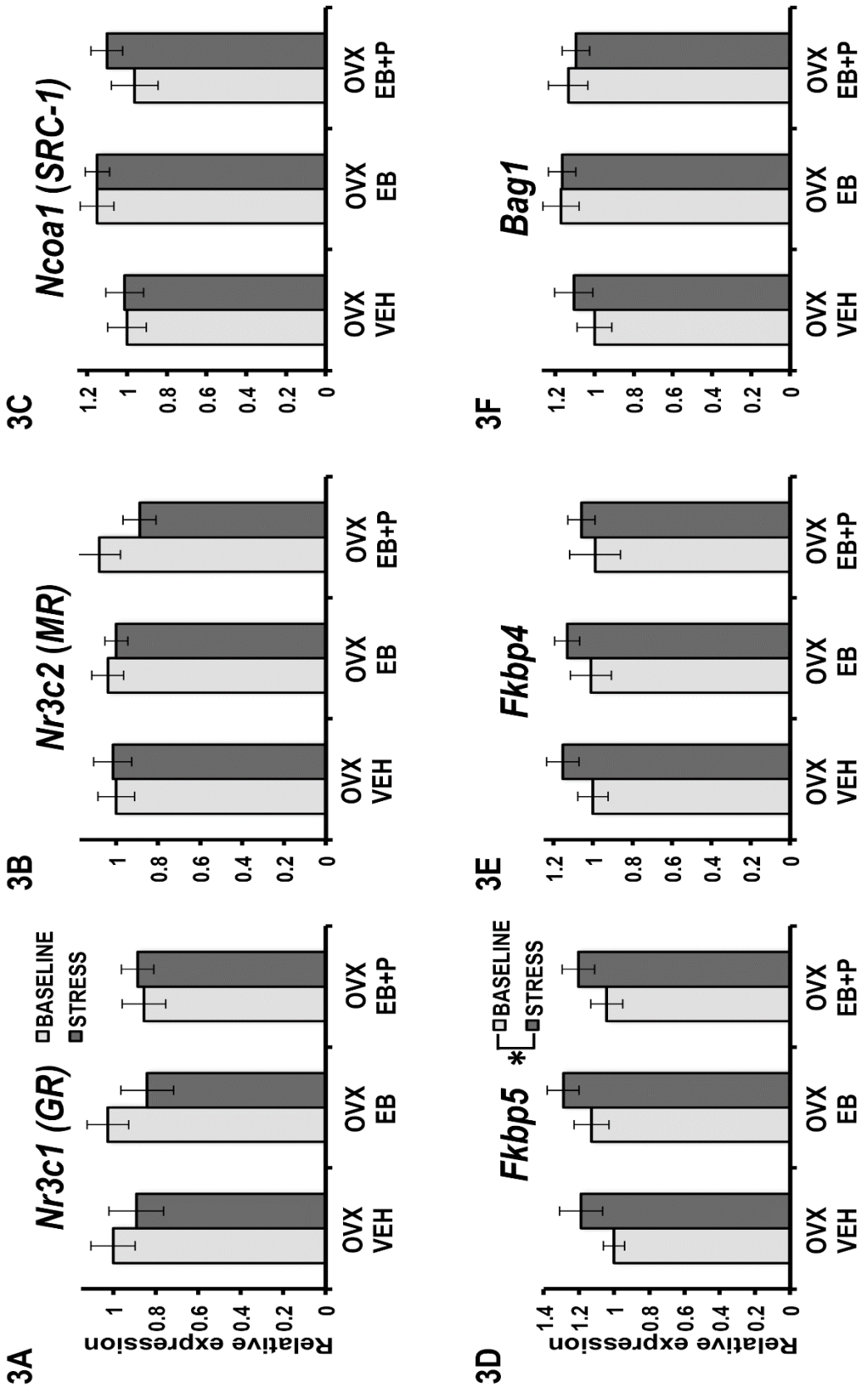
3A
Nr3c1 (GR)
BASELINE
STRESS
Relative expression
OVX VEH
OVX EB
OVX EB+P
3B
Nr3c2 (MR)
3C
Ncoa1 (SRC-1)
3D
Fkbp5
*
3E
Fkbp4
3F
Bag1

Figure 4-3. Relative mRNA expression (S.E.M.) of *Nr3c1* (A), *Nr3c2* (B), *Ncoa1* (C), *Fkbp5* (D), *Fkbp4* (E), and *Bag1* (F) at baseline (light-grey bars) and 90 minutes after 30 minutes of restraint stress (post-stress) with 1.0 set to OVX-VEH at baseline. * indicates a significant effect of time-point.

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Chapter 5: General Discussion and Conclusions

Summary of thesis findings and their contribution to the field of neuroendocrinology

The overarching goals of this thesis were to examine developmental shifts in HPA function and its regulation by gonadal hormones, and to investigate the mechanisms underlying these changes. In Chapter 2, I provided evidence of developmental shifts in the hormonal profile (testosterone, corticosterone, and progesterone) of intact male rats during the adolescent period. Pre-pubertal adolescent (P35) males had lower basal concentrations of testosterone compared with post-pubertal adolescent (P45) and adult males (P75) and, after acute stress, concentrations decreased in the adolescent groups and increased in adults, indicating developmental changes in gonadal regulation by stressors between puberty and adulthood (see Fig. 2-1A). The stress-induced decrease and increase of testosterone in adolescent and adult males, respectively, has been reported elsewhere (Foilb, Lui, & Romeo, 2011; Gomez, Houshyar, & Dallman, 2002; Romeo, Lee, Chhua, McPherson, & McEwen, 2004).

Consistent with previous studies (Klein & Romeo, 2013; Romeo, 2010; Romeo, Bellani, & McEwen, 2005), I found that pre-pubertal adolescents released more corticosterone and progesterone in response to acute stress compared with adult males (see Fig. 2-1B/C). Post-pubertal adolescents, for which there are fewer investigations, also had greater stress-induced release of progesterone compared with adults, but did not differ in corticosterone (concentrations were intermediate to that in pre-pubertal adolescents and adults). Therefore, there may be age-related changes in enzymes involved in adrenal hormone synthesis that restricts corticosterone production and bias

precursors (e.g., progesterone) during the post-pubertal period. Nevertheless, there was a trend for greater release of corticosterone at P45 than at P75, which is consistent with reports that HPA responses continue to mature between P40 and P60 (Foilb et al., 2011; Gomez et al., 2002). I have also shown that post-stress concentrations of progesterone and corticosterone were correlated in all age groups examined (see Fig. 2-1D), which has been previously reported in adults (Hueston & Deak, 2014), and likely reflects individual differences in general adrenocortical output.

The pronounced HPA response in adolescence can be attributed to differences at each level of the axis (Klein & Romeo, 2013), and likely reflects greater drive and reduced negative feedback. Protracted release of ACTH and corticosterone in response to stress points to negative feedback as a basis for age-related differences, and is not likely related to steroid metabolism (Schapiro, Percin, & Kotichas, 1971; Vazquez, 1998) or to circulating CBG concentrations (Romeo et al., 2006). Consistent with the idea that feedback is maturing in adolescence, GR agonists were less effective at suppressing HPA responses in pre-pubertal adolescents compared with adults (Goldman, Winget, Hollingshead, & Levine, 1973; Vazquez & Akil, 1993). Other studies had investigated GR protein and mRNA expression as a source for age-related differences and found little to no change in stress-related brain regions (e.g., PVN, pituitary, hippocampus, mPFC) (Dziedzic, Ho, Adabi, Foilb, & Romeo, 2014; Romeo, 2013; Romeo et al., 2008; Vazquez, 1998). Nevertheless, there may be age-related differences in GR's affinity for corticosterone or its activity when bound that mediates poor negative feedback in pre-pubertal adolescents.

In Chapter 2, I investigated GR expression in the cytosolic and nuclear compartments of hippocampal tissue at baseline and after stress as a proxy for translocation. The hippocampus was selected based on its rich expression of GR and because it is a major forebrain region involved in negative feedback (Herman, McKlveen, Solomon, Carvalho-Netto, & Myers, 2012; Mizoguchi et al., 2009). I also measured gene expression of corticosteroid receptors (*Nr3c1*, *Nr3c2*) and of co-chaperones (*Fkbp5*, *Fkbp4*, *Bag1*) that modulate steroid receptor activity. Consistent with previous studies in rats and mice (Dziedzic et al., 2014; Romeo, Kaplowitz, Ho, & Franco, 2013), I found that baseline expression of hippocampal GR (cytosolic + nuclear) was similar across age groups (see Fig. 2-2A-2E), although others have reported a decrease between P35 and ~P100 (Meaney, Sapolsky, & McEwen, 1985). In rats that underwent restraint stress, cytosolic GR decreased and nuclear GR increased and the change was more pronounced at P35 than at P75, providing novel evidence that GR translocation in the hippocampus is not blunted during adolescence and thus, is not a basis for immature feedback. Greater GR translocation in pre-pubertal males was likely because of their greater release of corticosterone in response to stress or because they had elevated expression of *Fkbp4* mRNA compared with adults (*Fkbp4* codes for the GR pro-translocation co-chaperone, Fkbp52) (see Fig. 2-1C and 2-4D).

After 30 minutes of recovery from restraint stress, pre-pubertal adolescents had greater GR expression (cytosolic and nuclear) than did adults, but because we could not compare this time-point with earlier ones, it is unclear whether the age-related difference reflects greater synthesis in adolescents, degradation in adults, or both. Because GR is auto-regulated (glucocorticoids suppress their own receptor targets) via hyper-

phosphorylation-induced ubiquitination and proteasomal degradation (Vandevyver, Dejager, & Libert, 2012) and via decreased mRNA expression (Burgess & Handa, 1993; Romeo et al., 2008), greater degradation in adulthood than in adolescence is a more likely a source for age-related differences. However, others have reported no change in total GR protein in pre-pubertal adolescents and adults 30 minutes after stress, relative to baseline (Dziedzic et al., 2014), and a comparable decrease in GR was observed after 5 days of corticosterone treatment (Meaney et al., 1985). An alternative explanation is that GR may have been preferentially trafficked to another cellular compartment (e.g., the cell membrane) in adults relative to pre-pubertal adolescents, but as we only examined cytosolic and nuclear expression, this remains unknown.

I found no age-related differences in the mRNA expression of corticosteroid receptors (*Nr3c1* and *Nr3c2*) or of the other co-chaperones examined (*Fkbp5* and *Bag1*) (see Fig. 2-4). Consistent with a previous study (Romeo et al., 2008), stress reduced *Nr3c1* (GR) mRNA expression in adolescent and adult males to the same extent, despite age-related differences in stress-induced release of corticosterone as well as in GR translocaton (as shown in the present study). These finding suggests a dissociation between the magnitude of an HPA response and the downstream changes in gene transcription. Another dissociation between stress response and changes in gene expression was also observed in the present study for the expression of *Fkbp5* mRNA, which was up-regulated after stress to a similar extent in all age groups. An avenue for future research will be to examine age-related differences in the regulation of gene expression (e.g., chromatin remodelling, expression of co-factors, activators, and repressors) after stress or corticosterone treatment, as the basis for the dissociation.

In Chapter 3, I investigated whether age-related differences in circulating concentrations of testosterone account for differential corticosterone responses to acute stress; in adult males testosterone reduces HPA activation and facilitates a return to basal titers (Goel, Workman, Lee, Innala, & Viau, 2014). I have provided evidence that age-related differences in HPA responses to acute stress persist when males of each age are OCX and given a comaparable dose of testosterone, consistent with the results of a previous study (Romeo, Lee, Chhua, et al., 2004). Additionally, I have shown that the effect of testosterone on stress-induced corticosterone release changes during adolescence, and provide evidence of developmental shifts in reactivity during the post-pubertal period that depends on the presence of testosterone. Specifically, testosterone had no effect on corticosterone release at P35, increased concentrations of corticosterone at P45, and tended to reduce concentrations at P75 (see Fig. 3-2C, middle panel). Moreover, in OCX males that received testosterone, corticosterone responses were more pronounced at P35 and P45 than at P75, whereas in those that did not get testosterone, P35 males responded more strongly than did P45 and P75 males. Together, these findings reveal developmental shifts in HPA function and its regulation by gonadal hormones.

Stress increased Fos expression in the PVN, which was used as a marker of neuronal activation (see Fig. 3-3D). There was nearly an effect of age ($p = 0.064$), which would be consistent with previous studies showing greater expression of Fos in the PVN of pre-pubertal adolescents compared with adults (Lui et al., 2012; Novak, Parfitt, Sisk, & Smale, 2007; Romeo et al., 2006; Viau, Bingham, Davis, Lee, & Wong, 2005). There was no effect of testosterone on Fos expression, however, which was expected in adults based on previous studies showing an androgen-induced decrease in its protein and

transcript expression (Lund, Hinds, & Handa, 2006; Lund, Munson, Haldy, & Handa, 2004; Viau, Lee, Sampson, & Wu, 2003; Williamson, Bingham, Gray, Innala, & Viau, 2010).

When the HPA axis is activated, AVP is released alongside CRF from neurons that originate in the medial parvocellular PVN and project to the median eminence. I measured AVP expression in the PVN based on previous evidence for androgen-induced suppresion of its mRNA or peptide expression (Evuarherhe, Leggett, Waite, Kershaw, Atkinson, et al., 2009; Seale, Wood, Atkinson, Harbuz, & Lightman, 2004; Viau et al., 2003; Viau & Meaney, 1996). Consistent with the literature, testosterone reduced AVP, and the effect was present before and after stress and in all age groups (see Fig. 3-3E). These findings indicate that pre-pubertal adolescent males are responsive to androgenic actions in the PVN, despite testosterone not affecting post-stress concentrations of corticosterone at this age. These findings also indicate that the age-related differences in the effect of testosterone on stress-induced release of corticosterone do not involve differences in AVP production.

Instead, I predicted that age-related differences in testosterone's regulation of corticosterone release may involve differences in testosterone's conversion to various metabolites, and I attempted to answer this using AR and ER antagonists in the presence of testosterone or DHT. The results indicated a similar, albeit non-significant, pattern of results to what we found in experiment 1 (see Fig. 3-2C, middle panel, and Fig. 3-5A), but no significant effects involving age, DHT treatment, or receptor antagonists on post-stress concentrations of corticosterone. The lack of an age-related difference conflicts with findings from Chapter 2 and Chapter 3 (experiment 1) and may reflect the fact that

experiment 2 invovled repeated injections prior to acute stress and plasma collection. The lack of an effect is not likely related to ineffective androgen implants because both testosterone and DHT reduced post-stress concentrations of progesterone, an effect that was reversed by the AR antagonist flutamide (see Fig. 3-5C and 3-5D). Therefore, I hypothesized that different findings in experiment 2 may relate to the stressful nature of our manipulations prior to measuring stress reactivity. Age-related differences in HPA function have been examined in response to a homotypic and heterotypic stress (Lui et al., 2012; Romeo et al., 2006; Romeo, Karatsoreos, Jasnow, & McEwen, 2007), but how stress history modulates the effect of gonadal hormones on HPA function requires invetigation.

In Chapter 4, I examined ovarian hormone regulation of HPA function in adult females. Although several studies have examined developmental shifts in ovarian regulation of HPA function during adolescence (Evuarherhe, Leggett, Waite, Kershaw, & Lightman, 2009; Romeo, Lee, & McEwen, 2004), the mechanisms remain unclear, and investigations in adults may provide insights necessary to understanding age-related differences. I have shown that OVX dampens the corticosterone response to stress compared with the response in gonadally-intact females, and that estradiol administered alone or in combination with progesterone potentiated the response to that in gonadally-intact females (see Fig. 4-1A). These findings are consistent with the majority of studies that investigated ovarian hormones regulation of HPA function (Green & McCormick, 2016; Handa & Weiser, 2014). I also investigated GR translocation in the hippocampus based on previous reports that estradiol impedes negative feedback (Burgess & Handa, 1992; Carey, Deterd, de Koning, Helmerhorst, & de Kloet, 1995; Viau & Meaney, 1991;

Weiser & Handa, 2009) and found that OVX increased the expression of cytosolic GR relative to other groups, which may be a basis for blunted feedback in estradiol treated females (see Fig. 4-1C). In experiment 2, I replicated the stimulatory effect of estradiol on corticosterone release, but when I examined corticosterone recovery 45 and 90 minutes after restraint stress all groups had approached baseline concentrations, which means I did not see evidence of delated recovery (see Fig. 4-2). Progesterone partially mitigated the effect of estradiol, which is consistent with a previous report (Viau & Meaney, 1991), but conflicts with the lack of an effect reported by others (Ferreira-Silva, Helena, Franci, Lucion, & Anselmo-Franci, 2009; Young, Altemus, Parkinson, & Shastry, 2001) and with results of experiment 1, which involved only one post-stress time-point.

Estradiol has been reported to modulate mRNA of corticosteroid receptors and co-activators *in vivo* (Burgess & Handa, 1993; Mitev, Wolf, Almeida, & Patchev, 2003) and co-chaperones *in vitro* (Malviya et al., 2013), which led us to investigate their expression along side GR translocation. I found no evidence for estradiol-induced changes in mRNA expression for any of our genes of interest (see Fig. 4-3). I did, however, find that stress increased *Fkbp5* expression regardless of treatment in females, just as it did for males of different ages in Chapter 2. Together, these findings indicate that OVX reduces HPA function, which may involve greater inhibitory tone by increasing the availability of GR protein.

Implications and outstanding questions

Studies included in this thesis have contributed to the field by replicating and expanding past findings regarding developmental shifts in HPA function and its

regulation by gonadal hormones during adolescence and early adulthood. Nevertheless, the mechanisms that govern some of the changes remain unclear and require future investigation. The studies contained in this thesis and answers to oustanding questions that are proposed below are important because they can enhance our understanding of factors that drive adolescent development, and that allow for long-term organization of cognition, affect, and behaviour by environmental influences during this time.

Adolescence is a transitional period between childhood and adulthood that is characterized by changes in cognition and behaviours, such as increased exploration, risk-taking, and emphasis on peer relationships (McCormick, Mathews, Thomas, & Waters, 2010; Schulz & Sisk 2016). These changes reflect maturation of underlying neural circuits and in the hormonal milieu (e.g., HPA and HPG output) during this time. The reorganization and fine tuning of these circuits that occurs in preparation for adulthood is made possible by heightened plasticity, which also renders adolescents vulnerable to insults (McCormick et al., 2010). Consistent with this notion, chronic stress during adolescence has long-lasting effects on cognition, mood, endocrine function, and social behaviour that are observable in adulthood (Green, Barnes, & McCormick, 2013; Green & McCormick, 2013; McCormick & Green, 2013).

Greater activation of the HPA axis in response to stress during adolescence may also explain why this age group is more sensitive to the programming effects of stress compared with adults, but more research is needed to tease apart the source of adolescent vulnerability. In Chapter 2, I report that GR translocation in response to stress was greater in pre-pubertal adolescents compared with adults, which corresponded with their greater release of corticosterone and expression of *Fkbp4* mRNA. Although it was meaningful to

examine age-related differences in GR translocation *in vivo*, and in response to a perceived stressor, it would be worthwhile to examine age-related differences in translocation when corticosterone concentrations are held constant. This constancy could be accomplished by adrenalectomy and systemic or central administration of corticosterone and would have the advantage of identifying developmental changes in stress signaling under constant conditions. Moreover, future studies should investigate differences in gene expression in males from each age group that are given a comparable dose of corticosterone that mimics stressful conditions. The studies proposed above may uncover age-related differences in stress signaling and may identify targets of stress that are differentially regulated as a function of age, both of which could underscore adolescent vulnerability.

Age-related differences in progesterone release after stress, which were presented in Chapter 2 and elsewhere (Romeo et al., 2005), suggest that progesterone should also be held constant when examining developmental shifts in HPA function (e.g., PVN activation and ACTH release) and gene transcription; progesterone may also contribute to the reduction in HPA function after a stressor exposure (Hueston & Deak, 2014). Such investigations may provide insight into how progesterone modulates HPA function and reveal mechanisms underlying developmental shifts in the response to acute and chronic progesterone exposure.

A related question that remains is, are there advantages to having a pronounced and prolonged stress response during adolescence, provided that the HPA axis is not activated too often or in relation to a traumatic event? During the stress-hyporesponsive period, which in rats occurs during the first two weeks of life, HPA responses to stressors

are blunted, presumably to protect the brain from insult during a period of rapid development (Meaney, Sapolsky, & McEwen, 1985; Naninck, Lucassen, & Bakker, 2011). Adolescence, which is also a sensitive period of development, however, involves pronounced HPA responses to challenges. One explanation is that greater HPA responses during adolescence may help to consolidate emotionally-salient events (Barbayannis et al., 2017), which may prepare adolescents for adulthood by shaping expectations for related-outcomes and coping strategies in the face of future challenges. Therefore, advancing our understanding of factors that promote vulnerability during adolescence may concomitantly elucidate the mechanisms that promote adaptive maturation of function, and refine how we think about adolescent development in general.

Advancing our understanding of adolescent development and how the brain responds to stressors during this time could also provide insight into the etiology of various psychopathologies (e.g., anxiety and depression), which tend to emerge during adolescence (Altemus, Sarvaiya, & Epperson, 2014). Psychopathologies tend to be associated with a history of stressful experiences and those afflicted typically have HPA dysregulation. For example, individuals that suffer from depression typically have elevated concentrations of resting cortisol and blunted HPA feedback (Naninck, Lucassen, & Bakker, 2011). Evidence from animal models indicates that chronic stress experienced in adolescence causes an increase in depressive and anxiety-like behaviour in adulthood (McCormick & Green, 2013), which suggests that the association in humans is not explained by a greater tendency to report stressful events by depressed individuals. Therefore, investigations into maturation of HPA function in adolescence may provide insight into mechanisms related to psychopathology as well as potential therapeutic

targets. Moreover, because there are well-documented sex differences in the prevalence and symptom severity of psychopathologies (women are approximately twice as likely to suffer from depression than are men) that emerge during adolescence (Naninck et al., 2011), investigations into HPA and HPG interactions may also elucidate targets.

We have provided evidence for developmental shifts in gonadal regulation of HPA function in adolescence and shown that pre-pubertal adolescents are sensitive to androgens, as evidenced by reduced AVP expression in the PVN. These findings indicate that testosterone's actions depend on the developmental context, and challenge the notion that pre-pubertal animals are unresponsive to gonadal hormones. Therefore, the pre-pubertal brain may already be capable of being organized by gonadal hormones, and consistent with this notion, evidence in hamsters shows that testosterone can masculinize reproductive behaviour before adolescence (Schulz & Sisk, 2016). Thus, maturation of HPG function at puberty and the greater conversion of testosterone to estradiol at this time likely dictates when the second window for gonadal organization occurs (perinatal being the first). A consequence of the greater conversion of testosterone to estradiol in the post-pubertal adolescents may be a pronounced response to stressors.

Although pre-pubertal males do not have adult-like levels of androgens, investigations into gonadal regulation of HPA function during this time will likely advance our understanding of adolescent development. Future studies are needed to elucidate the underlying mechanisms governing age-related changes in HPG regulation of HPA function remain unclear. The contribution of potential receptor targets of gonadal hormone metabolites on HPA function should be investigated based on evidence for changing enzyme expression in adolescence (Lephart & Ojeda, 1990); I attempted to do

this in Chapter 3, but saw little evidence for changes in corticosterone release. Nevertheless, this is only the first study to examine the effects of testosterone on HPA function in adolescence, as well as the receptors mediating testosterone's actions; others have characterized targets for gonadal regulation in adulthood (Handa & Weiser, 2014; Lund et al., 2006). Additionally, I only examined concentrations of corticosterone and progesterone at one post-stress time-point, and differences are likely to present in other HPA related measures or in the input from upstream regions (e.g., peri-PVN, BNST, mPOA, and hippocampus).

A limitation of the study that examined receptor targets for testosterone (and potential metabolites) was the stressful nature of our manipulations. Nevertheless, research into the effect of gonadal regulation of chronic stress responses is particularly important for understanding stress-related psychopathologies and provides greater translational relevance for humans; the human stress response is initiated in the context of a life-time of prior exposure to stressful events. Moreover, it is worth understanding how chronic stress regulates gonadal status across development; myself and others (Foilb et al., 2011) have shown that acute stress suppresses testosterone release in adolescents and increases its release in adults. In adults, repeated exposure to stress suppresses gonadal function, but only a couple studies have examined the effects of chronic stress on gonadal function in adolescents and adults (Gomez et al., 2002; Gomez, Manalo, & Dallman, 2004). Many studies have examined the effects of chronic stress in adolescence on adult measures of social behaviour, learning and memory, and mood (Green & McCormick, 2013; McCormick & Green, 2013), but it remains unclear whether adolescent sensitivity

is driven directly by stress or indirectly via changes in gonadal hormones that could disrupt adolescent development.

Answers to the above questions, coupled with the findings contained in this thesis, will enhance our understanding of adolescent development and neuroendocrine function. Moreover, because of the pleiotropic effects of glucocorticoid signaling and its involvement in health, these investigations may provide insight into the mechanisms that underlie dysfunction related to psychopathologies (e.g., anxiety and depression) and elucidate markers of vulnerability. In Chapter 4, I investigated gonadal regulation of HPA function in adult females to lay the groundwork for future investigations into the mechanisms underlying female maturation of HPA during adolescence. Studies on female rodents are critically important because the majority of research on HPA maturation has been conducted on males, and because sex differences often emerge. Lastly, although investigations in rodents do not always translate to humans, they are essential for understanding the principles that govern neuroendocrine function and how it changes across development, and provide a framework as well as targets for studies in humans, which are currently lacking.

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Appendix A

Animal Care Committee (ACC)
Chair –Fiona Hunter, PhD 905.688.5550 ext 3394
ACC Veterinarian – Alistair Ker, DVM 905.227.7644
Animal Care Committee Coordinator – Dayle Carlson, RMLAT 905.688.5550 ext 5820

Date: Aug 28, 2014

Dear Dr. McCormick and Mr. Green,

Your "Animal Use Protocol (AUP)" entitled:

Examining the expression and function of FK506 binding proteins 51 and 52 on stress reactivity before, during, and after puberty in male Long Evans rats.

has been approved by the Animal Care Committee.

This approval expires in one year on the last day of the month.
The number for this project is **AUP # 14 - 08 - 01**.
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED: **240 male long evans rats**

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

~~Fiona Hunter~~, Chair of ACC
Jean Hampson, Vice-Chair

THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY AND IS SUBJECT TO POST APPROVAL MONITORING.

ALL UNEXPECTED MORTALITIES MUST BE REPORTED TO ANIMAL CARE SERVICES STAFF IMMEDIATELY.

Appendix B

*AUP Renewal Application Teaching and Research Page **4** of **4***

AUP No. 14-08-01 Renewal 1 of 3

Original Title of Project: Examining the expression and function of FK506 binding proteins 51 and 52 on stress reactivity before, during, and after puberty in male rats.

SECTION IV PRINCIPAL INVESTIGATOR, TECHNICIAN AND/OR STUDENT INVESTIGATOR DECLARATION

Signatures reflect commitment to abide by the following principles for the duration of the animal project proposed:

a. All animals entered into this project will be treated in a humane manner, in accordance with the principles and guidelines of the CCAC such as are stated in the "Guide to the Care and Use of Experimental Animals".
b. This renewal accurately describes all proposed animal use. It will be kept current and will be modified only after obtaining approval of the Animal Care Committee (ACC).
c. All procedures will be carried out by well-trained and experienced personnel who are competent in the use of recognized techniques.
d. All procedures, which may cause pain or discomfort have been technically improved and/or minimized in such a way so that the expected results will be obtained with a minimum of discomfort to the animals.
e. The protocol number assigned by the ACC to this submission will be used when ordering animals and these animals will be used only for the project described.

Cheryl McCormick — 29/10 2015
Principal Investigator's Name and Signature — Date (dd/mm/yyyy)

Matt Green — 28/10 2015
Lead Technician / / Student Investigator's Name and Signature — Date (dd/mm/yyyy)

Travis Hodges — 29/10/2015
Technician Name and Signature (if applicable) — Date (dd/mm/yyyy)

Jonathan Simone — 29/10/2015
Technician / Student's Name and Signature (if applicable) — Date (dd/mm/yyyy)

Marina de Lima Marcolin — 29/10/2015
Technician / Student's Name and Signature (if applicable) — Date (dd/mm/yyyy)

ACC APPROVAL

Wendy Ward — 2/11/2015
ACC Chair's Name and Signature — Date (dd/mm/yyyy)

Comments:

Appendix C

Animal Care Committee (ACC)
Chair –Fiona Hunter, PhD 905.688.5550 ext 3394
ACC Veterinarian – Alistair Ker, DVM. 905.227.7644
Animal Care Committee Coordinator – Dayle Carlson, RMLAT 905.688.5550 ext 5820

Date: June 5, 2014

Dear Dr. McCormick and Mr. Green,

Your "Animal Use Protocol (AUP)" entitled:

Comparing Testosterone's Regulation of HPA Function Before and After Puberty in Male Long Evans Rats

has been approved by the Animal Care Committee.

This approval expires in one year on the last day of the month.
The number for this project is **AUP # 14 - 04 - 05**.
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED: **112 male long evans rats**

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

Fiona Hunter, Chair of ACC

THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY AND IS SUBJECT TO POST APPROVAL MONITORING.

ALL UNEXPECTED MORTALITIES MUST BE REPORTED TO ANIMAL CARE SERVICES STAFF IMMEDIATELY.

Appendix D

Animal Care Committee (ACC)
Chair –Fiona Hunter, PhD 905.688.5550 ext 3394
Clinical Veterinarians – Dr. Alistair Ker and Dr. Susan Warren
Animal Care Committee Coordinator – Dayle Carlson, RMLAT 905.688.5550 ext 5820

Date: June 15, 2015

Dear Dr. McCormick and Mr. Green,

Your "Animal Use Protocol (AUP)" entitled:

Examining the effects of testosterone on HPA function in male Long-Evans rats before, during, and after puberty

has been approved by the Animal Care Committee.

This approval expires in one year on the last day of the month.
The number for this project is **AUP # 15 - 03 - 01**.
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED: 252 male Long Evans rats

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

Jean Hampson, Acting Chair of ACC

Final approval is conditional upon arrangements being made for Post Approval Monitoring including a veterinarian for orchiectomy procedure on the first group of rats to receive surgery.

THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY AND IS SUBJECT TO POST APPROVAL MONITORING.

ALL UNEXPECTED MORTALITIES MUST BE REPORTED TO ANIMAL CARE SERVICES STAFF IMMEDIATELY.

Appendix E

*AUP Renewal Application Teaching and Research Page **4** of **5***

AUP No. 15-03-01

Original Title of Project:

Examining the effects of testosterone on HPA function in male Long-Evans rats before, during, and after puberty. Renewal 1 of 3

SECTION IV PRINCIPAL INVESTIGATOR, TECHNICIAN AND/OR STUDENT INVESTIGATOR DECLARATION

Signatures reflect commitment to abide by the following principles for the duration of the animal project proposed:

a. All animals entered into this project will be treated in a humane manner, in accordance with the principles and guidelines of the CCAC such as are stated in the "Guide to the Care and Use of Experimental Animals".
b. This renewal accurately describes all proposed animal use. It will be kept current and will be modified only after obtaining approval of the Animal Care Committee (ACC).
c. All procedures will be carried out by well-trained and experienced personnel who are competent in the use of recognized techniques.
d. All procedures, which may cause pain or discomfort have been technically improved and/or minimized in such a way so that the expected results will be obtained with a minimum of discomfort to the animals.
e. The protocol number assigned by the ACC to this submission will be used when ordering animals and these animals will be used only for the project described.

June 17/16

Marina Marcolin

Cheryl McCormick — / / June 15/16
Principal Investigator's Name and Signature — Date (dd/mm/yyyy)

/ /
Alternate Investigator's Name and Signature — Date (dd/mm/yyyy)

Matt Green — / / June 15/16
Lead Technician / Student Investigator's Name and Signature (if applicable) — Date (dd/mm/yyyy)

Jonathan Simone — / / June 15/16
Technician / Student's Name and Signature (if applicable) — Date (dd/mm/yyyy)

Travis Hodges — / / June 15/16
Technician / Student's Name and Signature (if applicable) — Date (dd/mm/yyyy)

As Departmental Chair, I agree to fund animal-related expenses incurred by this teaching protocol up to a maximum amount of $

/ /
Departmental Chair's Name and Signature (Teaching Renewals Only) — Date (dd/mm/yyyy)

ACC APPROVAL

Dana J. Smith — 22/6/2016
ACC Chair's Name and Signature — Date (dd/mm/yyyy)

Comments:

Valid exemption for testosterone required.

Appendix F

Brock

Animal Care Committee (ACC)
Chair –Fiona Hunter, PhD 905.688.5550 ext 3394
Clinical Veterinarians – Dr. Alistair Ker and Dr. Susan Warren
Animal Care Committee Coordinator – Dayle Carlson, RMLAT 905.688.5550 ext 5820

Date: October 3, 2014

Dear Dr. McCormick,

Your "Animal Use Protocol (AUP)" entitled:

Ovarian hormonal regulation of HPA function and effects on expression and function of FK506 binding proteins 51 and 52 in Long Evans rats.

has been approved by the Animal Care Committee.

This approval expires in one year on the last day of the month.
The number for this project is **AUP # 14 - 09 - 03**.
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED: 96 female Long Evans rats

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

Fiona Hunter, Chair of ACC

THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY AND IS SUBJECT TO POST APPROVAL MONITORING.

ALL UNEXPECTED MORTALITIES MUST BE REPORTED TO ANIMAL CARE SERVICES STAFF IMMEDIATELY.

Appendix G

Brock

Animal Care Committee (ACC)
Chair –Fiona Hunter, PhD 905.688.5550 ext 3394
Clinical Veterinarians – Dr. Alistair Ker and Dr. Susan Warren
Animal Care Committee Coordinator – Dayle Carlson, RMLAT 905.688.5550 ext 5820

Date: May 26, 2016

Dear Dr. McCormick and Mr. Green,

Your "Animal Use Protocol (AUP)" entitled:

Ovarian hormonal regulation of hypothalamic-pituitary-adrenal function and gene expression in Long Evans rats

has been approved by the Animal Care Committee.

This approval expires in one year on the last day of the month.
The number for this project is **AUP # 16 - 04 - 01**.
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED: 48 female long evans rats

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

Fiona Hunter, Chair of ACC

THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY AND IS SUBJECT TO POST APPROVAL MONITORING.

ALL UNEXPECTED MORTALITIES MUST BE REPORTED TO ANIMAL CARE SERVICES STAFF IMMEDIATELY.

Appendix H

Health Canada / Santé Canada

Healthy Environments and Consumer Safety Branch / Direction générale, Santé environnementale et sécurité des consommateurs

Address Locator 0300B
Ottawa ON K1A 0K9

Your file / Votre référence

Our file / Notre référence

2017-03-07

HC6-53-40-51

Cheryl McCormick, PhD
Department of Psychology
Faculty of Social Sciences
Brock University
1812 Sir Isaac Brock Way
St. Catharines ON L2S 3A1

Authorization 24134.11.10STK2/AMD/EXT
Expiry Date: 2018-03-07

"15-03-01: Examining the effects of testosterone on HPA function in male Long-Evans rats before, during and after puberty"

Dear Dr. McCormick:

In response to your recent request for an extension of your previous exemption issued on February 12, 2016 to use a controlled drug, the following exemption is being granted to you pursuant to section 56 of the *Controlled Drugs and Substances Act* (CDSA) on the basis that such exemption is necessary for a scientific purpose.

With respect to the controlled drug listed below, and in accordance with the protocol(s) specified above, you are hereby exempted from the application of the following provisions of the CDSA and Part G of the *Food and Drug Regulations* (FDR) as they apply to the following activities:

- Subsections 5(1) and 5(2) of the CDSA as they apply to the administration of a controlled drug and to the possession of the controlled drug for that purpose, if such possession and administration is in accordance with the research protocol submitted.

The exemption herein is only applicable if you comply with the following conditions:

(1) You may only possess the controlled drug listed below, up to the quantity indicated:

Substance Name	Maximum Quantity
Testosterone	4 g

Canada

…/2

Appendix I

(2) You may only possess the controlled drug listed above which you indicated as the remaining quantity from your previous exemption;

(3) You may only be in possession of the controlled drug listed above if such possession is for the following purpose:

Note: purpose must be marked with an "X" to be applicable.

[ ] to use *in vitro* in accordance with the research protocol submitted;

[X] to administer to animals in accordance with the research protocol submitted.

(4) The controlled drug must be used solely for the purpose marked above;

(5) You are required to maintain records with respect to your possession and use of the controlled drug in accordance with section G.06.002 of Part G of the *Food and Drug Regulations*. You shall make such records available to the Minister or an inspector upon request. Specifically, you must keep and retain for a period of two years from the making of such record, the following information:

- the name and address of the supplier from whom the controlled drug was received;
- the brand name of the substance, or if it does not have a brand name, the generic name of the substance;
- the quantity, the strength per unit and the date on which the substance was received; and
- details of the use of the controlled substance.

(6) You must safeguard the controlled drug from theft in a satisfactory manner, and report any theft or loss within 10 days of its discovery to the Compliance and Monitoring Division, Office of Controlled Substances, by calling: 613-952-2177 or email: OCS_Reporting_Rapporter_BSC@hc-sc.gc.ca;

(7) Colleagues, assistants, technicians, etc. participating in the same project, who are under your direction and control, are also exempted to the same extent and for the same purpose as you are. You are responsible for any portion of the controlled drug listed above that is used by such an individual;

(8) If the project is terminated before the expiry of this exemption, you must notify the Office of Controlled Substances of your remaining inventory of the controlled drug kept under this exemption;

(9) You must inform the Office of Controlled Substances if you leave the project before it is completed or terminated, so that a formal transfer of responsibilities for the controlled drug to another researcher may be provided;

Appendix J

(10) You must obtain prior authorization from the Office of Controlled Substances before using any controlled drug remaining from this project, within the context of another project, whether that project is your own or that of another researcher.

(11) You are responsible for the destruction of any unused or expired controlled drug. The destruction must be witnessed by a member of your research staff who is working on the same research project as specified in this exemption, and who works under your direction and control. The method of destruction used must alter or denature the controlled drug in such a way as to make it non-recoverable and thus make their consumption improbable or impossible. You are required to keep and retain for a period of two years from the date of the making of the record, the following information:

- the name, strength per unit, and quantity of any controlled drug to be destroyed;
- the date of destruction; and
- the reason for destruction.

Immediately following the destruction, you and the witness are required to sign and print your names on a joint statement indicating that you witnessed the destruction and that the controlled drug destroyed has been altered or denatured to such an extent that its consumption has been rendered impossible or improbable.

You shall make such records available to the Minister or an inspector upon request.

(12) You will permit entry by any inspector designated under the CDSA, at any reasonable time to ensure compliance with the exemption and in accordance with subsections 31(2) to (9) and section 32 of the CDSA. For the purpose of the inspection, you will permit the inspector to exercise all the powers found in paragraphs 31(1)(a) to (i).

This exemption expires on the earliest of the following four dates:

- the date of formal transfer of responsibilities for the controlled drug kept under this exemption;
- the date the quantity authorized by this exemption has been entirely used;
- the date on which this exemption is replaced by another exemption; or
- March 7, 2018.

If you plan to continue working on the same project beyond the expiry date of this exemption, and you have any controlled drug remaining in your inventory, you must request an extension or a new exemption before the expiry date of this exemption.

Appendix K

Failure to comply with the terms and conditions of this exemption may, among other things, result in immediate suspension of this exemption, and ultimately, in its revocation.

If you have any further questions regarding this exemption, please do not hesitate to contact the Exemptions Section at 613-954-8246.

Yours sincerely,

Mark Kozlowski
Acting Manager
Authorizations Division
Office of Controlled Substances